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Early Blood Leucocyte Changes in Mice and Guinea Pigs Following X-Irradiation and Stress Caused by Operative Manipulations

By

A. Forssberg B Tribukait and K. J Vikterlöf

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Abstract

Forssberg, A., B. Tribukait and K. J Vikterlöf *Early blood leucocyte changes in mice and guinea pigs following X-irradiation and stress caused by operative manipulations* Acta physiol. scand. 1961 *52* 1—7 — Rapid shifts in the relative frequencies of the white cells of animals may occur following injection e.g. of toxins or other agents, radiation, but even mechanical manipulations (Jacobson 1954, Craddock 1960) The present work demonstrates the complexity of the reactions and the species differences between mice and guinea pigs following X-radiation and the manipulations (sham irradiation) A detailed survey (Table I—III) shows, that in mice eosinophils decrease following sham and X-radiation, that monocytes fall significantly by sham treatment but not furtheron after radiation, that rods increase continuously in number by application of sham treatment and X-rays. In these and other respects guinea pigs show a different behaviour bearing witness to the multiformity of the reactions.

Leucocytes in general are apt to be released into the circulation from myeloid marrow at short notice. The capillary beds of various tissues seems to afford another reservoir (Harris 1959 Osgood 1954) The highly differentiated mode of reaction of the various leucocytes and the species differences is however not easily accounted for on the notion of a simple mechanism regulating the flow to and from the circulating blood.

The habitual action of ionizing radiation on the white cell picture of the circulating blood is that of causing a general decrease [illegible] which becomes manifest some [illegible] post-irradiation. The magnitude and duration of this decrease is dose dependent and may serve as an indication of a radiation influence of the organism in practice. Preceding this stage a period of very rapid concentration changes during the first hours was noted by many workers, although detailed analyses do not seem to have been performed.

On the other hand the white cells are very mobile and a flow to and from the circulation is caused by a variety of measures including injections of chemicals, hormones among others. It may well be that the irradiation effects are mediated through the release of some such substances. The complexity of the early white cell reactions was also found in our preliminary trials and particularly the species differences between mice and guinea pigs. This led us to a general survey of the early radiation reactions, including also the simultaneous [illegible] caused by the mechanical manipulations of the animals.

Material and method

Male guinea pigs were obtained from a [illegible] days at the institute before the experiment. The weight of the animals varied between 190–250 g but within each series of experiments within ± 10 g from the average weight. [illegible] main experiments. They were carried out on irradiation experiment and [illegible] as far as possible [illegible]

Irradiations were given at 200 kV and 15 mA [illegible] The X-ray beam [illegible] during the exposure. In the case of guinea pigs the total dose was [illegible] from the dorsal [illegible] and [illegible] from above [illegible] variations [illegible] through [illegible] irradiation [illegible] variations [illegible]

Preliminary trials [illegible] the mechanical manipulations of bringing the animal into [illegible] and giving a sham irradiation [illegible]

[illegible] manipulation and irradiation [illegible] the animals [illegible]

[illegible]

[illegible]

Table I Differential counts of mice leucocytes under varying experimental conditions: animals sacrificed directly taken from their cages (1) subjected to manipulations in connection with irradiation (2) given 205 r and 595 r (3, 4) given pentobarbitone injection and irradiation in anesthesia as compared to directly sacrificed mice (5) animals continuously transported in their permanent cages (perm.) and given 185 as compared to directly sacrificed mice (6)

– number of animals in each series.

Series		n	tot. leuco.	rods	segm.	eos.	mono.	lympho.
1	direct	29	7,202	6	738	70	135	6,233
2	sham	22	2,675	19	595	18	46	1,997
3	205	27	2,689	42	897	12	62	1,678
4	595	10	2,968	69	1,371	7	66	1 453
5	direct	8	8,462	8	743	52	127	7,532
	pentob.	7	7,190	33	2,926	36	166	4,029
	pentob. + 185	8	4,816	13	2,445	23	83	2,252
6	direct	8	6,516	—	626	43	134	5,713
	perm.	8	4,927	2	528	48	56	4,293
	perm. 185	8	3,795	2	690	26	56	3,025

Table II Differential counts of guinea pig leucocytes. Experimental conditions in relevant respects similar to table I

Series	n	tot. leuco.	rods	segm.	eos.	mono.	lympho.
direct	10	4,439	14	1 123	21	102	3,166
sham	29	3,400	9	843	13	118	2,404
5	10	3,374	15	1 115	32	139	2,062
24	10	5,832	11	3,112	34	253	2,398
50	11	7,863	26	5,054	29	444	2,247
200	15	5,087	97	3,395	44	273	1,257
400	13	3,106	169	2,214	23	221	465

stained samples were performed on 500 cells. Rod forms, segmented cells, eosinophils, basophils, monocytes and lymphocytes were scored. Basophils, which occurred in low concentrations and very irregularly in guinea pigs and are practically absent in mice, have not been tabulated.

Results

Earlier work has demonstrated that the peripheral leucocyte concentration increases during the first post-irradiation hours. Provided the doses are well below sublethal level, normal or nearly normal, values appear again after about 6 hours. A secondary rise is often seen at a later stage. Detailed analyses of the very early steps of this reaction does not seem to have been undertaken.

Table III Significance of reactions of mice and guinea pigs towards sham treatment and X-radiation. P-values > 0.05 are not recorded

Measured	Mice	Guinea pigs
sham effects, decrease of:		
eos.	< 0.001	–
mono.	< 0.001	–
lympho.	< 0.001	–
increase of: rods	< 0.02 > 0.01	–
X-ray effects, increase of:		
rods	< 0.001	< 0.001
segm.	0.001	< 0.001
eos.	–	< 0.001
mono.	–	< 0.001
decrease of eos.	< 0.001	–

Our preliminary trials indicated that the divergent reactions of the various leucocytes and the species differences were most pronounced 2—4 hours after the X-ray dose and about equally well expressed. Thus, in the main experiments irradiated and sham treated animals were sacrificed 2 and 4 hours after the treatment and the results were pooled in the tables.

Mice The data are summarized in Table I and II and the significance of some main results, particularly in regard to the species differences, in Table III Data with P values > 0 05 have not been considered in the discussion and in Table III

Comparing the two groups direct and sham (series 1 and 2) it appears that the manipulations concomitant to the preparation for irradiation causes a very pronounced effect which is most impressive in lymphocytes. The fall of lymphocytes accounts mainly for the reaction of circulating total leucocytes. It should be noted that the lymphocytes proved to be the only cell group which by all treatments decreased in number mice and guinea pigs alike, whereas varying behaviour characterized all other cell forms. Sham treatment of mice increased rods but decreased eosinophils significantly Application of X-rays of the order of 200 r (3) did not cause distinct effects, but when increasing the dose to about 600 r (4) a trend becomes obvious, namely that rods increase and eosinophils decrease continuously by application of irradiation on top of the sham manipulation. The statistical significance of both these trends was proved by the method of correlation of ranks (LEVY and PREIDEL 1959) It may be noted that monocytes do not react towards irradiation and that a slight increase of segmented is barely perceptible after the higher dose

Apart from these series two minor experiments were performed in the first of which we ventured an attempt to reduce the sham effects by pentobarbitone

injection prior to irradiation series (5). This failed, and besides a significant increase of rods, which also occurs in the sham group, a significant outflow of segmented cells was also found. There is a general trend of a decrease of all leucocytes by irradiation under anesthesia although not statistically significant in this small series. Finally the possibility was considered that a cautious transport of the animals may reduce the stress effect (series 6). The counts indicate a considerable reduction of the manipulation effects, but a trend similar to that found in the sham series (2) is still obvious.

The possibility that younger mice, weight 9—10 g may react in a more pronounced manner was tried in a single experiment of one group of controls and a group given 200 r and analyzed 2 hours post-irradiation. The reaction of these young mice was, however entirely similar to that of full grown animals.

Guinea pigs. The material is rather limited and does not allow for definite conclusions in all instances. However it is safe to state that the guinea pigs differ *in several respects, both qualitatively and quantitatively from mice.* (Table II and III.)

In the first place the manipulations in connection with the irradiations did not cause such marked effects as by mice. The trend of a slight fall of all leucocyte cells except for monocytes is not significant in the present material. Studies of the X-ray effects were performed over a wider range of doses since guinea pigs are on the whole more radiosensitive than mice and show definite effects at least down to doses of the order of 25 r. The X-ray reactions of rods is in common with mice; moreover the tendency of a lymphocyte decrease, which is suggested in the mice experiments, becomes higly significant in guinea pigs following doses of 200—400 r. In other respects the reaction of guinea pigs differ from mice. There occurs a radiation induced increase of eosinophils, the dose dependence of which is not quite clear. An increase of segmented cells and monocytes in the irradiated animals is also highly significant. The data suggest however, that the X-ray effect may reach a maximum at doses somewhere round 50, whereas higher doses cause another superimposed, effect of diminishing the outflow of leucocytes.

Discussion

A diversity of reactions of the white blood cells in regard to the early release to, and disappearance from, the circulation has been found in the two species. Differential counts at such early stages are rather scarce in the literature. Some data are however provided by HULSE (1960) on rats given 100, 600 and 5 000. This author irradiated the animals in their ordinary cages, thus with a minimum of disturbances, and his controls nearly conform to our group "direct". As in our mice material radiation caused a decrease 4 hours after the dose of eosinophils, similarly monocytes showed no early influence and rods and segmented cells increased, although only after 600 and 5 000 r. There occurred in

rats also a considerable decrease of lymphocytes already after 100 r This finding sheds some light on the reactions of lymphocytes in our mice material. Evidently the manipulations *per se* depleted the lymphocytes so that no further reduction by irradiation is achievable.

The rapid increase of circulating leucocytes seems partly to be due to a rapid mobilization of mature, or nearly mature, cells from mother tissues, preferably the marrow HARRIS (1960) made studies of bone marrow and blood from irradiated guinea pigs It appeared that at least segmented cells are discharged *from the marrow in amounts which roughly accounts for the early increase of* this cell group in the blood. Detailed analyses in regard to other cells and the dose dependence were however not performed.

The bone marrow on the other hand acts as a depot for lymphocytes which leave the circulation (HARRIS 1959 and YOFFEY 1960) There seems to be a reversed reaction in the sence that when granulocytes leave the marrow lymphocytes are taken up. The general fall of circulating lymphocytes by all sorts of treatments in our case is likely explained in this way Other tissues may also serve as a depot for segregated cells. Following treatment with adrenaline and histamine this was the case for lungs, spleen and intestines (AMBRUS and AMBRUS 1959)

Leucocytes are very motile cells which according to OSGOOD (1954) are moving to the sites where they are best needed. From quantitative counts of the distribution of total leucocytes in blood, bloodforming tissues and other tissues this author arrived at a figure of about 70 per cent in other tissues" against only about 0 3 per cent in the blood A vast reservoir is thus present here for rapid mobilization.

Another noteworthy feature is the complex relationship of sham treatment and irradiation, which may act synergistically or independantly of each other The fact that radiation sensitivity and manipulation sensitivity do not always run parallel was clearly demonstrated in a work by ALBAUM (1960) on the reactions of whole body X rayed rabbits serum enzymes. Early concentration changes *occurred in the serum and from* 14 enzymes assayed no less than 11 showed sham effects. Some of these showed additional X ray effects, whereas others were unchanged by the irradiation. There were also enzymes which neither reacted towards irradiation nor towards sham manipulations. A wide reaction spectrum, similar to that of cells, *is consequently also to be found on the mole*cular level. Since many of the enzymes which displayed different reaction had about the same molecular weight the particle size as such is not a decisive factor in the mechanism of controlling flow to and from the circulation.

Perhaps the most reasonable hypothesis is that the rapid and differentiated shifts of the localization of the various leucocytes is mediated by hormonal action or by the release of substances like histamine An early rise of circulating total leucocytes is *e g* caused by adrenaline injection in humans. (LUCIA, LEONARD and FALCONER 1937) In dogs early eosinopenia occurred after the

injection of ACTH, adrenaline or histamine (LAST *et al.* 1950). Numerous other observations demonstrate similar actions on the white cell distribution.

That hormones may interfere with irradiation effects in a complex way on the chemical level has been visualized in other connections. Adrenaline and ACTH was, for example, found to change the rate of resorption and the exhalation of $^{14}CO_2$ from injected $NaH^{14}CO_3$ in mice (FORSSBERG and HEVESY 1955).

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References

ALBAUM, H. G., Serum enzymes following whole-body radiation in the rabbit. *Radiation Res.* 1960. *12.* 186—194.

AMBRUS, C. M. and J. L. AMBRUS, Regulation of the leukocyte level. *Ann. New York. Acad. Sci.* 1959. *77.* 445—486.

CRADDOCK, C. G., Production and distribution of granulocytes and the control of granulocyte release. Ciba Foundation Symposium on Haemopoiesis, *J. & A. Churchill.* (Lond.) 1960 237—261.

FORSSBERG, A. and G. HEVESY, Effect of X-rays and hormones on resorption rate of injected $NaH^{14}CO_3$. *Amer. J. Physiol.* 1955. *180.* 325—330.

HARRIS, P. F., The correlation between bone marrow activity and blood neutrophil levels from quantitative studies in irradiated guinea-pigs. *Brit. J. exp. Path.* 1959. *40.* 589—600.

HOLM, E. V., The total white cell count of the blood as an indicator of acute radiation damage and its value during the first few hours after exposure. *J. clin. Path.* 1960. *13.* 37—41.

JACOBSON, L. O., The hematologic effects of ionizing radiation. Radiation Biology edited by A. Hollaender. *McGraw-Hill Book Co. Inc.* (New York) 1954. *1.* 1029—1090.

LAST, J. H., P. JORDAN, L. PITESKY and E. BOND, The eosinophil response. Immediate vs. Delayed eosinopenia. *Science* 1950. *112.* 47—49.

LEVY, H. and E. E. PREIDEL, Elementary statistics. *Th. Nelson and Sons Ltd.* (Lond.) 1959 pp. 74.

LUCIA, S. P., M. E. LEONARD and E. H. FALCONER, The effect of the subcutaneous injection of adrenaline on the leukocyte count of splenectomized patients and of patients which certain diseases of the hematopoietic and lymphatic systems. *Amer. J. med. Sci.* 1937. *194.* 35—43.

OSGOOD, E. E., Number and distribution of human hemic cells. *Blood* 1954. *2.* 1141—1154.

YOFFEY, J. M., The evolution of the lymphomyeloid tissues. Ciba Foundation Symposium on Haemopoiesis, *J. & A. Churchill* (Lond.) 1960. 1—42.

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Histamine Release Elicited by Extracts from Ascaris Suis — Influence of Oxygen Lack and Glucose

By

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Abstract

Diamant B. *Histamine release elicited by extracts from Ascaris suis — influence of oxygen lack and glucose.* Acta physiol. scand. 1961. *52.* 8—22. — In guinea pigs intraperitoneal injection of *Ascaris* extract in doses effective in rats neither released histamine into the peritoneal fluid nor "disrupted" mast cells in the mesentery. *In vitro* studies on *Ascaris*-induced histamine release from rat lung tissue showed the release to be dependant on pH, incubation time and concentration of extract. Under oxygen lack, the histamine release by *Ascaris* extract diminished *to about 9 % of that in oxygen, but increased in the presence of glucose to* about 80 % of the values found in the presence of both oxygen and glucose (5.6 mmole/l). With oxygen alone 60 % of this value was released. The enhancement of histamine release caused by glucose in nitrogen as well as oxygen suggests that it is dependant on enzymatic processes yielding high energy compounds.

During the past decades various theories have been presented concerning the mechanism of histamine release and mast cell "disruption". Several of the theories maintain that enzymatic processes are involved in these reactions. Thus, Parrot (1942), Mongar and Schild (1957), Chakravarty (1959) and Diamant and Uvnäs (to be published) have shown that oxygen lack inhibits the histamine release as well as the mast cell "disruption". Further evidence has been presented by Junqueira and Beiguelman (1955), Mongar and Schild (1957), Högberg and Uvnäs (1958, 1960), Mousatché and

PROVOUST DANON (1958) CHAKRAVARTY (1959) UVNÄS and THON (1959 1960) and others, who have shown that histamine release and mast cell disruption depend on pH and temperature and that various enzyme inhibitors act as blocking agents. These investigations concern the action of antigen on sensitized tissues and synthetic histamine liberators on non-sensitized tissues as well as on isolated mast cells *in vitro* Compound 48/80 has been investigated far more extensively than other synthetic histamine liberators.

In addition to the synthetic histamine liberators there are several of biologic origin. A very active liberator can be extracted from *Ascaris suis* an eelworm occurring in the hog ROCHA E SILVA and GRAÑA (1946) showed that *Ascaris* extracts, when injected into dogs, produced histamine shock indistinguishable from anaphylactic shock" *Ascaris* extract has also been found to release histamine *in vitro* from perfused cat paws and to disrupt" mast cells of rat mesentery *in vitro* (HÖGBERG, THURESSON and UVNÄS 1956 HÖGBERG *et al.* 1957) *It has been suggested that the mast cell disruption" caused by Ascaris* extract involves enzymatic processes (UVNÄS *et al.* 1960) Our knowledge of these enzymatic processes is still, however imperfect, though the blocking of mast cell disruption" by such metabolic inhibitors as dinitrophenol thyroxine, cyanide etc. indicates that the disruption" is an energy requiring process.

The present investigation mainly concerns the histamine releasing activity of *Ascaris* extracts, tested in vitro on rat lung tissue under various conditions. The aim was to evaluate the role of glucose in *in vitro* histamine releasing *reactions, as judged from the effect of Ascaris extract on rat lung tissue under the* influence of nitrogen and oxygen respectively

Materials and methods

Ascaris Extract

The preparation of *Ascaris* extract has been reported elsewhere. (UVNÄS *et al.* 1960) *The activity of an extract, which heated to 100° C for 20 min in 50* ethanol, is fairly stable when stored at 4 C. The amounts of extract referred to are valid for dialysed acid extract dissolved in an equal volume of ethanol, the dry weight of 1 ml of which amounted to 6—10 mg, depending on the stock solution used. Three different stock solutions were used in the present experiments and are referred to in the following as *Ascaris* extract I, II and III.

Prior to all experiments the required amount of *Ascaris*-ethanol solution was dried *in vacuo* to eliminate the alcohol. The dry extract was then dissolved in a buffered isotonic solution (see below) and the pH was adjusted to between 6.9 and 7.2. In the experiments with isolated rat lung tissue the concentration of the *Ascaris* extract prepared always amounted to 10 times of that finally wanted. In the experiments with intraperitoneal injection of *Ascaris* extract into rats and guinea pigs *in vivo* the *Ascaris* extract was further diluted with buffered isotonic solution to the required concentration.

Buffered Isotonic Solution

A solution containing NaCl (154 mmole/l) KCl (2.7 mmole/l) and $CaCl_2$ (anhydrous, 0.9 mmole/l) buffered with Sorensen phosphate buffer (67 mmole/l) 10 % v/v was

used as incubation medium in the experiments with isolated rat lung tissue. The pH of the medium was kept between 6.9 and 7.2.

Intraperitoneal Injection of Ascaris Extract

10 ml *Ascaris* extract, treated as described above, was injected intraperitoneally into rats (weighing 150—200 g) and guinea pigs (weighing 350—400 g) of both sexes. 10 min after the injection the animals were anesthetized with ether and killed by exsanguination. The abdomen was opened and the intraperitoneal fluid removed by pipette. The fluid was then centrifuged (2,500—3,000 r. p. m. for 5 min) and stored at — 20° C until assayed for histamine content.

Three pieces were cut from different areas of the mesentery and were fixed and stained in a water solution containing 4 % formaldehyde and 0.1 toluidine blue (NORTON 1954). At microscopic examination of the mesenteric specimens, a mast cell was considered to be "disrupted" if 3 or more granules were detected outside the cell. A hundred cells were counted from each specimen from which the "disruption" was calculated as percentage.

Experiments with Isolated Rat Lung Tissue

The method used corresponds, in general, with CHAKRAVARTY's (1959) modification of UNGAR and PARROT's (1936) method. Male and female rats weighing 250—450 g were used. After lightly anesthetizing the rat with ether the thorax was opened and the heart rapidly excised. This procedure precluded aspiration of blood into the lungs. Following exsanguination, the lungs were removed from 3—5 animals and stored in the buffered isotonic solution. All visible bronchi were removed from the lobes. Each lung lobe was divided into 6—12 approximately equal parts, depending on the number of samples required for the experiment. To assure a more or less uniform distribution of the rat lungs, all samples were prepared so as to include a portion of each lung lobe. The samples were blotted dry with filter paper and weighed, each sample usually weighing 500—600 mg (in no instance less than 400 mg). The samples were cut into smaller pieces (1—2 mg) and carefully washed with buffered isotonic solution. They were finally incubated in small Erlenmeyer flasks and rocked in a Warburg apparatus at 37° C. The time required for preparation of the lung tissue samples prior to incubation was 1 / —3 hours.

The lung samples were uniformly incubated for 15 min in the incubation medium before addition of the *Ascaris* extract. In the experiments on the influence of oxygen and nitrogen on histamine release the gases were bubbled through the solutions via syringe needles during this 15-minute period. In order to avoid foaming when the *Ascaris* extract was added to the samples, the needles were withdrawn above the surface of the solutions for the rest of the experiment. In the experiments where the effects of glucose as well as magnesium chloride were investigated, these substances were present in the buffered isotonic solution during preparation and incubation of the lung tissue samples.

Unless otherwise stated the *Ascaris* extract was kept in contact with the lung samples for 20 min. The incubation fluid was then filtered through cotton wool. After centrifugation, the pH of the incubation fluid was determined electrometrically and the fluid was stored at — 20° C for subsequent histamine assay.

In all experiments the *Ascaris* extract, concentrated as described above, was added in a volume of 0.1 ml/100 mg lung tissue. The final volume of incubation fluid amounted to 1 ml/100 mg lung tissue. In the various experiments the final concentration of *Ascaris* extract amounted to 0.5 ml/ml incubation fluid if not otherwise stated.

For extraction of the histamine remaining in the lung samples after the incubation

Table I. Effect of intraperitoneally injected Ascaris extract I in rats and guinea pigs

Species	ml Ascaris extract per ml buffer solution injected intraperitoneally	"Disrupted" mesenteric mast cells, in %	Histamine base per ml peritoneal fluid, in μg
Rat	0.2	49	0.50
	0.5	81	0.93
	0.3	100	1.70
	Control	0	< 0.01
Guinea pig	0.5	0	0.03
	0.5	0	0.03
	Control	0	< 0.01

10 ml injected into each animal. In controls buffered isotonic solution without Ascaris extract I was used.

1 ml of the buffered isotonic solution was added per 100 mg lung tissue. Extraction was done by heating the separate samples for 30 min at 100° C on a water bath. The fluids were then filtered through cotton wool, centrifuged and stored at −20° C until assayed for histamine.

The total histamine content varied between 2.4—9.1 μg histamine base per g of lung tissue in the different experiments.

In order to verify that all the remaining histamine was extracted by the above procedure, an additional extraction on random samples was performed ad modum FELDBERG and TALESNIK (1955), i.e. by boiling over an open flame for 3 min with 8 ml buffer solution and 2 ml N HCl per g of lung tissue. Following centrifugation and neutralization, the amount of histamine remaining was never more than 3 % of the total histamine content of the lung samples. This small amount was disregarded when computing the histamine release as per cent of the total histamine content.

In order to investigate if the extracted histamine was inactivated when exposed to 100° C for 30 min, identically treated samples were extracted for 5 and 30 min respectively with and without addition of N HCl. Since in all cases the amount of histamine extracted was found to be the same, this could be precluded.

The variation of Ascaris-induced histamine release in duplicate samples amounted on an average to ± 1.4 % of the total histamine content (range ± 0.1—± 5.0) as judged from 7 duplicate tests from different experiments where the histamine release varied between 30—40 % of the total histamine content.

Histamine Assay

Histamine was assayed on atropinized (1.5×10^{-4} M atropine sulphate) guinea pig ileum by comparing the contraction induced by a standard histamine dihydrochloride solution with at least 4 contractions of each experimental sample. The Ascaris extract in the concentrations used caused no contraction of guinea pig ileum and did not influence the histamine response. Mepyramine (10^{-4}—10^{-7} M) completely abolished the contractions produced by randomly selected experimental samples.

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For extraction of he histamine remaining in the lung samples after the incubation

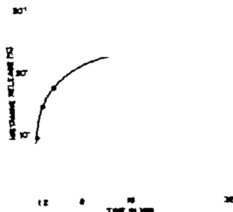


Fig. 2. Time course of *Ascaris*-induced histamine release from rat lung tissue. 0.75 ml *Ascaris* extract I per ml incubation fluid was used. Release values computed in % of the total histamine content. Spontaneous release deducted from all values.

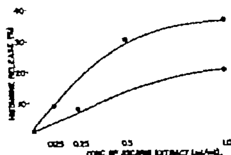


Fig. 3. Dose response. •—•—•—• = *Ascaris* extract II (stored 3 months at 4° C) •—•—•—• = *Ascaris* extract III (freshly prepared). The histamine release computed in % of the total histamine content. Spontaneous release deducted from all values.

About 50 % of the releasable histamine was liberated within 1 min after the addition of *Ascaris* extract. It seems clear that histamine release does not increase after 20 min, which has been the incubation time used in the present experiments.

Dose Response

Fig. 3 shows the histamine release caused by various concentrations of *Ascaris* extract II and *Ascaris* extract III. *Ascaris* extract II had been stored 3 months at 4° C while *Ascaris* extract III was freshly prepared. The distribution of the values are too large to permit detailed conclusions but the graph clearly shows the difference in histamine releasing activity of the 2 stock solutions of *Ascaris* extract tested.

In the experiments shown in the following figures, *Ascaris* extract III with a final concentration of 0.5 ml/ml incubation fluid was used throughout.

The Role of Oxygen

Fig. 4 shows that oxygen lack, produced by incubation of the lung samples under nitrogen, reduced the histamine release caused by *Ascaris* extract as

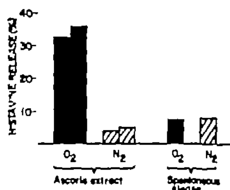


Fig. 4. Effect of nitrogen and oxygen on the histamine release elicited from rat lung tissue by *Ascaris* extract III (0.5 ml/ml incubation fluid). The variation of duplicate tests and the spontaneous release are shown. The spontaneous release is deducted from *Ascaris*-induced histamine release. All release values expressed in % of total histamine content.

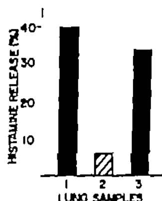


Fig. 5. Histamine releasing effect of *Ascaris* extract III (0.5 ml/ml incubation fluid) reincubated under oxygen (sample 3) after previous incubation under nitrogen (sample 2). Sample 1: Histamine release under oxygen without previous incubation of the extract under nitrogen. All values computed in % of the total histamine content. The spontaneous release is deducted from the release values of samples 1 and 2. Spontaneous release as well as the histamine release under nitrogen from sample 2 are deducted from the release value of sample 3.

compared with the histamine release under the influence of oxygen. The mean histamine release under nitrogen amounted to 5 % and under oxygen to 35 % of the total histamine content of the lung samples. This means that only 13 % of the histamine released under oxygen was liberated under nitrogen. It will also be noted that the spontaneous release with nitrogen approximated that with oxygen.

Table III A, on page 17, demonstrates the fall of histamine release under nitrogen compared with that under oxygen in 5 separate experiments. The *histamine release under nitrogen averaged about 9 % of that under oxygen* (range 2—14 %).

The possibility that the decreased amount of histamine found in the incubation fluids under nitrogen was due either to an inactivation of the *Ascaris* extract or to a decomposition of the released histamine could be ruled out as shown in Fig. 5 and Table II. Fig. 5 shows that *Ascaris* extract previously incubated with lung tissue under oxygen lack when reincubated with a new lung sample under oxygen released histamine in amounts comparable with those found in an oxygen control. This shows that the *Ascaris* extract was not inactivated under nitrogen when in contact with lung tissue. In addition

Table II. Differences in histamine release and total histamine content between lung samples treated with oxygen and nitrogen respectively

Experimental conditions	Experiment no. 1		2		3		4		5	
Oxygen	2.2	(4.9)	2.4	(5.1)	1.9	(3.9)	2.3	(4.3)	2.1	(4.9)
Nitrogen	0.6	(5.1)	0.6	(5.3)	0.4	(3.8)	0.8	(4.2)	0.6	(4.8)
Difference	1.6	(−0.2)	1.8	(−0.2)	1.5	(0.1)	1.5	(0.1)	1.5	(0.1)

Histamine release values without parentheses, total histamine content in parentheses. All values are given as µg histamine base per g rat lung tissue. In each experiment 0.5 ml *Ascaris* extract II per ml incubation fluid was used. No correction has been made for the spontaneous release.

nitrogen bubbled through an *Ascaris* extract for 10 min did not change the histamine releasing activity.

Table II shows the difference in histamine release caused by *Ascaris* extract under oxygen and nitrogen respectively, as well as in the total histamine content of the corresponding lung samples. Since for each experiment the total histamine content shows only negligible differences as compared with the release values, the diminished histamine release found under nitrogen cannot have been due to decomposition of the histamine released into the incubation fluid.

Enhancement of Histamine Release under Nitrogen and Oxygen

MOUSSATCHÉ and PROVOOST-DANON (1957 A) reported that aerobic anaphylactic histamine release from guinea pig lung tissue *in vitro* was stimulated by the presence of metabolites of Krebs cycle, an effect which they attributed to an increase of high energy compounds. This effect could not be demonstrated, however, when the histamine release was caused by compound 48/80 (MOUSSATCHÉ and PROVOOST-DANON 1957 B).

It was, therefore, of interest to investigate the effect of glucose (yielding high energy compounds through its aerobic as well as anaerobic breakdown) and of magnesium ions (constituting a co-factor in several of these enzymatic processes) on the histamine release elicited by *Ascaris* extract from rat lung tissue *in vitro* under various conditions. In the presence of glucose (anhydrous, 5.6 mmole/l) and $MgCl_2 \cdot 6H_2O$ (0.49 mmole/l) the inhibition of the histamine release was found to diminish markedly. Thus, under the influence of nitrogen the histamine release from 6 lung samples in the same experiment averaged 30 % of the total histamine content (range 27—33 %). From a 7th lung preparation under oxygen 34 % of the total histamine content was released in the presence of glucose and magnesium chloride in the above concentrations.

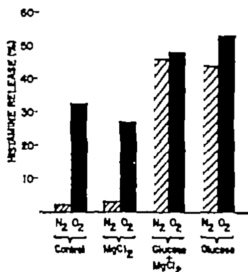


Fig 6. Effect of glucose (5.6 mmole/l) and $MgCl_2 \cdot 6H_2O$ (0.49 mmole/l) on the histamine release induced by *Ascaris* extract III (0.5 ml/ml inculation fluid) under oxygen and nitrogen. Control: Histamine release without influence of glucose or MgCl. Release values computed in % of total histamine content. The spontaneous release deducted from all values.

From this experiment however it was not possible to determine whether the presence of glucose or/and magnesium ions had caused the increased histamine release under nitrogen. A series of separate lung samples was, therefore prepared in pairs with buffered isotonic solution containing glucose or/and magnesium chloride in the above concentrations. Lung samples treated with a solution containing no glucose or magnesium chloride were used as controls. One of each pair of lung preparations was incubated under nitrogen and the other under oxygen in the same solution previously used for the preparation

It was found, as shown in Fig 6 that under nitrogen the histamine release caused by *Ascaris* extract was enhanced solely because of the presence of glucose. The histamine release from the lung samples under oxygen was also found to increase in the presence of glucose Thus, in this experiment 53 % of the total histamine content was released from the lung sample treated with oxygen and glucose. With oxygen, but without glucose the release decreased to 33 %. This means that about 60 of the histamine release under oxygen in the presence of glucose occurred under oxygen alone. Magnesium chloride in the concentration used had no demonstrable increasing effect on the histamine release under nitrogen or oxygen either alone or in conjunction with glucose

Table III B shows the histamine release caused by *Ascaris* extract under the influence of nitrogen in 4 different experiments with and without 5 6 mmole/l of glucose in the med um Without glucose as will be seen from the last column of Table III B, on the average only 2 / (range 0—5 %) was released as compared with the release when glucose was present On the other hand as shown in Table III C, representing 3 separate experiments, the histamine release under nitrogen in the presence of glucose averaged 82 (range 75—80) of that under oxygen n the presence of glucose

Table III. A. B. C. Influence of oxygen and nitrogen, with and without addition of glucose (5.6 mmole/l) on the in vitro histamine release elicited by Ascaris extract from rat lung tissue

A.

Expt. no.	Histamine release under the influence of		
	N_2 without glucose (in % of total histamine content)	O_2 without glucose (in % of total histamine content)	N_2 without glucose (in % of release under O_2)
1	4.5	34.4	13
2	6.0	44.9	13
3	0.3	14.8	2
4	5.6	40.4	14
5	1.9	34.8	5
			Mean: 9

B.

Expt. no.	Histamine release under the influence of		
	N_2 without glucose (in % of total histamine content)	N_2 with glucose (in % of total histamine content)	N_2 without glucose (in % of release with N_2 and glucose)
1	2.0	43.8	5
2	0.1	28.7	0
3	0.4	24.4	2
4	0.3	24.3	1
			Mean: 2

C.

Expt. no.	Histamine release under the influence of		
	N_2 with glucose (in % of total histamine content)	O_2 with glucose (in % of total histamine content)	N_2 with glucose (in % of release with O_2 and glucose)
1	30.0	34.2	88
2	30.1	40.3	75
3	43.8	53.6	82
			Mean: 82

The spontaneous release deducted from all values. Different stock solutions of *Ascaris* extract in amounts ranging from 0.5 to 1 ml/ml incubation fluid were used in the single experiments.

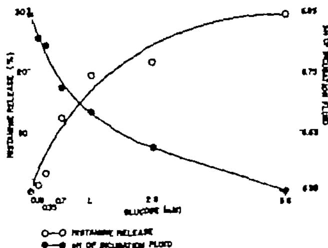


Fig 7 Effect of various concentrations of glucose (geometrically decreasing from 5.6–0 mmole/l) on the histamine release under nitrogen elicited by *Ascaris* extract III 0.5 ml/ml incubation fluid) from rat lung tissue as well as on the pH of the incubation fluid after incubation. Release values computed in % of total histamine content. The spontaneous release deducted from all values. The pH of the incubation media with and without glucose was 6.90 before incubation.

Effect of Varying Concentrations of Glucose on Histamine Release under Nitrogen

The effect of different concentrations of glucose on the histamine release under nitrogen is shown in Fig 7 The lung samples were prepared and incubated with media containing glucose in concentrations geometrically decreasing from 5 6 mmole/l Before the addition to the lung samples the pH of the incubation medium was 6 90.

The histamine release caused by *Ascaris* extract under nitrogen was found to increase with increasing concentrations of glucose. Following incubation of the lung tissue, the pH of the incubation fluids fell as the glucose concentration increased

Discussion

The species difference in histamine release as observed between rats and guinea pigs after intraperitoneal injection of *Ascaris* extract in this investigation is not surprising since similar differences have been reported for other histamine releasing agents (Feldberg and Mongar 1954 Mongar 1955) The absence of disrupted mast cells in guinea pig mesentery does not however preclude the possibility that mast cells had been affected by the *Ascaris* extract. Boréus (1960) showed that in guinea pigs histamine liberating agents caused disappearance of the mast cells without the manifest disruption" found in rats. Such a disappearance cannot be demonstrated with the method used in this investigation. The lack of detectable mast cell damage, however parallels the negative finding of histamine release. Guinea pigs, therefore are considered by the author to be insusceptible to *Ascaris* extract in the concentration used.

The histamine release from rat lung tissue elicited *in vitro* by *Ascaris* extract

is dependant on the pH of the incubation fluid, and maximal release values are obtained when the final pH is between 6.8 and 7.1. This accords with a previous investigation by UVNÄS *et al.* (1960) concerning the effect of *Ascaris* extract on mast cells *in situ*, for they noted a pH optimum around 7.0 for *Ascaris*-induced mast cell "disruption" in rat mesentery.

Judging from the time course of *Ascaris*-induced histamine release about 50 % of the releasable histamine was liberated into the incubation fluid within 1 min and the release was completed between 8 to 16 min after addition of *Ascaris* extract to the rat lung tissue. This is in accordance with the time course found by CHAKRAVARTY (1959) for anaphylactic histamine release from sensitized guinea pig lung tissue.

Different stock solutions of *Ascaris* extract when used under identical experimental conditions at the same concentration, show substantial variations in their histamine releasing capacity. This is attributed partially to unavoidable variations in the preparation procedure of the *Ascaris* extracts and partially to their inactivation during storage. Different degrees of inactivation are usually found after 2—3 months of storage with 50 % alcohol at 4° C. The inactivation occurs gradually. *Ascaris* extract used in experiments at varying intervals after preparation may thus give rise to variations in histamine release. Since, however, a single *Ascaris* extract was used in any given experiment, the comparisons presented refer to histamine release as elicited by the same stock solution.

The effect of oxygen lack on histamine release from lung tissue has previously been investigated by PARROT (1942), MONGAR and SCHILD (1957) and CHAKRAVARTY (1959). Using Tyrode solution as incubation medium, these authors showed that anaphylactic histamine release *in vitro* from guinea pig lung tissue requires oxygen. PARROT did not present figures but MONGAR and SCHILD reported an average of 32 % histamine release under oxygen lack (range 6—55 %) as compared with the release under air. CHAKRAVARTY found, with oxygen lack, a histamine release ranging between 14 and 29 % of the values found when oxygen was bubbled through the preparation during incubation. In rats, however, CHAKRAVARTY found no effect of oxygen lack on either anaphylactic or compound 48/80-induced histamine release from lung tissue incubated with Tyrode solution. MONGAR and SCHILD, furthermore, reported an increase under oxygen lack, of compound 48/80-induced histamine release from guinea pig lung tissue. Since guinea pig lung tissue is markedly insensitive to compound 48/80 and only responds when submitted to 100 to 1,000 times the concentration normally needed for histamine liberation in rat lung tissue (MONGAR and SCHILD 1957) the effect may be an unspecific one (BOREUS 1960). In the present experiments without glucose, the *Ascaris*-induced histamine release from rat lung tissue *in vitro* amounted, in oxygen lack, to only about 9 % of that found with oxygen. In earlier reports no information has been given concerning the presence of glucose in the Tyrode solution used. The fact that glucose greatly stimulates histamine liberation under oxygen lack, suggests

that previously reported variations in histamine release under oxygen lack may have been due, at least in part, to presence of glucose.

The acceleration of histamine release under oxygen lack in the presence of glucose was found to progress with increasing concentrations of glucose. At the same time the pH of the incubation fluid shifted more and more towards the acid side. Since *Ascaris*-induced histamine release was greatest at pH around 7.0 this shift towards the acid side cannot in itself be the cause of the increased histamine release.

The presence of 5.6 mmole/l of glucose was found to enhance histamine release elicited by *Ascaris* extract from rat lung tissue *in vitro* not only under nitrogen but also under oxygen. Thus far the maximal histamine release has been found when the lung tissue was exposed to *Ascaris* extract under oxygen in the presence of glucose. Considering this release as 100 %, the release under the influence of nitrogen and glucose was somewhat less, amounting to about 80 %; *with oxygen in the absence of glucose the release was reduced to about* 60 %, with nitrogen in the absence of glucose less than 10 % was released. These figures must be regarded as approximate, and no statistical analysis has been undertaken. Similar results have been found, however, regarding the effect of glucose, oxygen and nitrogen, in respect to anaphylactic histamine release from rat lung as well as from guinea pig lung tissue, and also in respect to the release caused by compound 48/80 from rat lung tissue (DIAMANT unpublished observations).

Anaphylactic histamine release from guinea pig lung tissue, according to MONGAR and SCHILD (1957) and CHAKRAVARTY (1959) involves enzymatic energy-requiring reactions. Their conclusions were drawn from the observation that lack of oxygen caused an inhibition of the histamine release *in vitro*. MOUSSATCHÉ and PROVOOST DANON (1957 A) showed, as stated above, that several of the metabolites in Krebs cycle (succinate, a-ketoglutarate and acetate) had a stimulating effect on the aerobic anaphylactic histamine release from guinea pig lung tissue. This was attributed to an increase of high energy compounds arising from oxidation of the metabolites. The current observation of an increased histamine release produced by the addition of glucose, supports the view that enzymatic reactions are involved and that high energy compounds, deriving from the enzymatic breakdown of glucose, may be of major significance. *It is apparent from the results that in the presence of glucose, even* when oxygen is lacking glycolysis may yield sufficient amounts of high energy compounds for histamine releasing reactions to occur.

In the present experiments, histamine was released from rat lung tissue under the influence of oxygen without addition of glucose, even though the experimental procedure involved treatment of the lung samples with glucose-free buffered isotonic solution for 1 $^{1}/_{2}$—3 hours prior to incubation. If, as suggested, high energy compounds are necessary for histamine release to occur it may be inferred that glucose is retained in the lung tissue in amounts sufficing for

aerobic conditions. However the possibility must not be discounted, as noted by Bing (1954) and Krebs (1957) that in the presence of oxygen, high energy compounds may be formed from sources other than carbohydrates. On the other hand, in the absence of oxygen under corresponding experimental conditions, the glucose presumably retained in the lung tissue may be either depleted or insufficient to yield enough high energy compounds during the 15 minute period of nitrogen exposure preceding contact with the *Ascaris* extract. This would suggest that the diminished histamine release found under the influence of nitrogen may be due to lack of glucose and consequently of high energy compounds. This is further supported by the finding (Diamant unpublished observations) that phlorizin under certain experimental conditions inhibits the stimulating effect of glucose on histamine release in the absence of oxygen.

High energy compounds are considered necessary for active transport mechanisms across cell membranes (Lehninger 1954). Such a mechanism might be involved in histamine releasing reactions in a way as yet undetermined. The final proof of an involvement of high energy compounds in histamine releasing reactions is, to judge from the relevant literature, still lacking although such a dependency has been further supported by the finding, that agents which uncouple phosphorylation simultaneously produce inhibition of histamine release (Mongar and Schild 1957, Mouschatché and Provoust Danon 1958, Westerholm 1960, Diamant and Uvnäs to be published). From the present investigation it is, however, tempting to emphasize the correlation between the metabolic situation in various conditions with glucose, oxygen and nitrogen and the histamine release. Furthermore, investigations on mast cells from rat peritoneum *in situ* by Diamant and Uvnäs (to be published) show that the "disruption" caused by compound 48/80 decreases under nitrogen and that this effect is counteracted by the presence of glucose.

Financial support for this investigation from Svenska Sällskapet för Medicinsk Forskning and Karolinska Institutet is gratefully acknowledged.

References

Bing, R. J. The metabolism of the heart. *Harvey Lect.* 1954—55. Series L. 27—70.

Boréus, L.-O. A comparison of mast-cell reactions in the rat, hamster and guinea pig. *Acta physiol. scand.* 1960. *49.* 251—260.

Chakravarty, N., The mechanism of histamine release in anaphylactic reaction in guinea-pig and rat. *Acta physiol. scand.* 1959. *48.* 146—166.

Diamant, B., Unpublished observations. 1960.

Diamant, B., B. Högberg, I. L. Thon and B. Uvnäs, Histamine release and mast-cell "fragmentation" produced by an agent isolated from Ascaris lumbricoides. *Acta physiol. scand.* 1957 *42.* Suppl. 145.

Diamant, B. and B. Uvnäs, To be published.

Feldberg, W. and J. Talesnik, Reduction of tissue histamine by compound 48/80. *J. Physiol.* (Lond.) 1953. *120.* 550—568.

Feldberg, W. and J. L. Mongar, Comparison of histamine release by compound 48/80 and octylamine in perfused tissues. *Brit. J. Pharmacol.* 1954. 9. 197—201

Högberg, B., G. Sudow, I. L. Thon and B. Uvnäs, The inhibitory action of a compound obtained from hip seeds (HSC) on the release of histamine and the disruption of mast cells produced by compound 48/80 and extracts from jellyfish (Cyanea capillata) and eel worm of swine (Ascaris lumbricoides). *Acta physiol. scand.* 1957. 38. 265—274

Högberg, B., G. Thufveson and B. Uvnäs, Histamine liberation produced in the perfused paw of the cat by 48/80 and extract from jellyfish (Cyanea capillata) and eel worm (Ascaris lumbricoides) from swine. *Acta physiol. scand.* 1956. 38. 135—144

Högberg, B. and B. Uvnäs, Inhibitory action of allicin on degranulation of mast cells produced by compound 48/80, histamine liberator from ascaris, lecithinase A and antigen. *Acta physiol. scand.* 1958. 41. 157—162

Högberg, B. and Uvnäs, B., Further observations on the disruption of rat mesentery mast cells caused by compound 48/80, antigen-antibody reaction, lecithinase A and decylamine. *Acta physiol. scand.* 1960. 48. 133—145.

Junqueira, L. C. U. and B. Beiguelman, Studies on the *in vitro* action of compound 48/80, formaldehyde condensation product, on rat mast cells. *Tex. Rep. Biol. Med.* 1955. 13. 69—75.

Krebs, H. A., Control of metabolic processes. *Endeavour* 1957. 16. 125—132.

Lehninger, A. L., Oxidative phosphorylation. *Harvey Lect.* 1953—54. Series XLIX. 176—215.

Mongar, J. L., Measurement of histamine-releasing activity. *Ciba Found. Symp. Histamine. J. and A. Churchill Ltd.* 1956. 74—91

Mongar, J. L. and H. O. Schild, Inhibition of the anaphylactic reaction. *J. Physiol.* (Lond.) 1957. 135. 301—319

Moussatché, H. and A. Provoust Danon, Histamina e metabolismo celular. I. Influência de alguns metabolitos sôbre a liberação de histamina na reação anafilática in vitro. *An. Acad. Bras. Cienc.* 1957. A. 29. XXX.

Moussatché, H. and A. Provoust Danon. The effect of succinate and malonate on the chemical release of histamine from guinea-pig lung slices. *Naturwissenschaften* 1957. B. 44. 637—638.

Moussatché, H. and A. Provoust Danon. Influence of oxidative phosphorylation inhibitors on the histamine release in the anaphylactic reaction *in vitro*. *Experientia* (Basel) 1958. 14. 414—415.

Norton, S. Quantitative determination of mast cell fragmentation by compound 48/80. *Brit. J. Pharmacol.* 1954. 9. 494—497

Parrot, J. L., Sur la réaction cellulaire de l'anaphylaxie. Son caractère anérobie. *C. R. Soc. Biol.* (Paris) 1942. 136. 361—362

Rocha e Silva, M. and A. Graña, Anaphylaxis-like reactions produced by Ascaris extracts. *Arch. Surg.* (Chicago) 1946. 52. 713—729

Ungar, G. and J. Parrot. Recherches sur le choc anaphylactique *in vitro*. Mise en liberté d'une substance active par le poumon isolé de cobaye sensibilisé. *C. R. Soc. Biol.* (Paris) 1936. 123. 676—678

Uvnäs, B. and I.-L. Thon. Isolation of biologically intact mast cells. Exp. Cell. Res. 1959. 18. 512—520.

Uvnäs, B. and I. L. Thon, Evidence for enzymatic histamine release from isolated rat mast cells. *Exp. Cell. Res.* 1960. In press.

Uvnäs, B., D. Diamant, B. Högberg and I. L. Thon, Mechanism of mast-cell disruption induced by a principle extracted from Ascaris suum. *Amer. J. Physiol.* 1960. In press.

Westerholm, B. In vitro release of histamine from cat skin by compound 48/80. *Acta physiol. scand.* 1960. 50. 41—48

Acta physiol. scand. 1961. 52. 23—31

From the Institute of Pharmacology, University of Oslo, Norway

Note on the Effect of Increased NaCl-Concentration on the Neuromuscular Transmission

Does Desensitization to Acetylcholine Take Place during Tetanus?

By

GUNNAR LILLEHEIL and KNUT NAESS

Received 5 January 1961

Abstract

LILLEHEIL, G. and K. NAESS. *Note on the effect of increased NaCl-concentration on the neuromuscular transmission.* Acta physiol. scand. 1961. 52. 23—31. — The possibility of desensitization of the motor end-plate to ACh during tetanus — as proposed by THESLEFF — and the possible role of this desensitization for production of Wedensky inhibition of the neuromuscular transmission, have been tested and are discussed.

As in THESLEFF's experiments increased concentration of NaCl has been used to obtain block of contraction and pure end-plate potentials. Mechanical registration of the contractions and extracellular recording of end-plate potentials were employed.

No signs of Wedensky-inhibition could be obtained by increasing the NaCl-concentration in the bath. Previous results indicate that the state of the motor end-plate after long sustained strong increase in the NaCl-concentrations must be rather unphysiological. The present results and also previous ones quoted in the discussion do not indicate any certain desensitization of the motor end-plate to ACh under physiological conditions. A mechanism of this type can probably not be of particular significance for the production of the very rapidly occurring Wedensky inhibition seen, for example, during curarization.

THESLEFF (1959) has recently published results obtained by use of increased concentration of NaCl (2.0—2.5 times the normal one) on the neuromuscular transmission in the rat phrenic nerve-diaphragm preparation. The purpose of

using this method was to reduce the mechanical movements of the muscle, making intracellular recording of end plate potentials (e.p.p.) possible without using tubocurarine or magnesium. It had previously been demonstrated (HOWARTH 1958) that twitch responses are abolished by use of hypertonic solutions (concentrated Ringer's solution or solutions made hypertonic with sucrose).

FATT and KATZ (1952) examined the effect of sodium ions on the neuromuscular transmission. They confined the investigation to the effect of decreasing concentrations of sodium, but also made a few observations on the effect of increased concentrations of sodium and addition of sucrose, thereby differentiating between the osmotic and the specific ionic effects. Some of their results will be discussed in relation to those of THESLEFF and the present ones.

THESLEFF used intracellular microelectrodes and tetanic stimulation followed by electrophoretically applied acetylcholine (ACh) and concluded according to his experimental results that "desensitization of end-plate receptors is produced by the transmitter agent and can account for the decline in amplitude of the successive end-plate potentials. Furthermore, the desensitization process is likely to be at least partly responsible for the neuromuscular transmission failure known as the Wedensky inhibition."

We are at present engaged in investigation of the Wedensky-inhibition produced during curarization, which according to our results must be a primarily presynaptic effect produced by tubocurarine (LILLEHEIL and NAESS 1960 and 1961).

For our further work and a more detailed discussion of the possible presynaptic effect of tubocurarine, the present investigation and ensuing discussion have been necessary.

Methods

Rat phrenic nerve-diaphragm preparation were prepared according to BÜLBRING (1946). Isotonic contractions were recorded as usual on a kymograph and e.p.p. were recorded on submerged preparations with a chlorinated silver electrode with diameter of 1 mm, isolated to the tip. The end-plate zone was located according to usual procedures (described in more detail by LILLEHEIL and NAESS 1961). Indirect supramaximal stimulation with impulses of 0.3 msec was used.

Oxygenated (+ 5 % CO_2) Tyrode's solution was used, and undissolved NaCl was added to the bath in an amount necessary to double the concentration of that salt. Decreased concentration of NaCl was used in a few experiments. Sucrose was added to make the solution isotonic. 1 VRU hyaluronidase (Invasin® Lundbeck") per 100 ml was used in one experiment to make the connective tissue of the preparation more permeable for the salt solution and to obtain the neuromuscular block as rapidly as possible.



Fig. 1 Single and tetanic contractions of rat diaphragm preparation produced by indirect supramaximal stimulation of phrenic nerve with frequencies of 1/sec and 50/sec.) Normal contractions. At arrow increase to twice the normal NaCl-conc. (2 × NaCl) b, c, d and, recorded 2, 7, 12 and 17 min afterwards. Drum stopped for 5 min at a_1, a_2 and a_3. At right usual not fully developed, Wedensky-inhibited contraction produced by curarization.

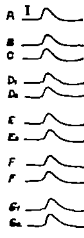


Fig. 2. Pure e.p.p. of rat phrenic nerve-diaphragm preparation recorded immediately after block had been obtained 60 min after change to 2 × NaCl. A, after single stimulation, B—G during tetanic stimulation with 50/sec. Time in sec for registration of potentials after start of stimulation B 0.5 C 1 D_1—D_2 4 E_1—E_2 8, F_1—F_2 12, G_1—G_2 16. Hyaluronidase used to increase the penetration of NaCl.

Results

Usual mechanical recordings of single and tetanic contractions produced under increased concentrations of NaCl are presented in Fig 1 It is quite obvious that no Wedensky-inhibition occur under these circumstances. Even with a frequency of 100/sec in a control experiment no sign of the slightest Wedensky inhibition was observed. The reduced tetanic contractions have on

using this method was to reduce the mechanical movements of the muscle, making intracellular recording of end-plate potentials (e.p.p.) possible without using tubocurarine or magnesium. It had previously been demonstrated (HOWARTH 1958) that twitch responses are abolished by use of hypertonic solutions (concentrated Ringer's solution or solutions made hypertonic with sucrose).

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Rat phrenic nerve-diaphragm preparation were prepared according to *BÜLBRING* (1946). Isotonic contractions were recorded as usual on a kymograph and e.p.p. were recorded on submerged preparations with a chlorinated silver electrode with a diameter of 1 mm, isolated to the tip. The end-plate zone was located according to usual procedures (described in more detail by LILLEHEIL and NAESS 1961). Indirect supramaximal stimulation with impulses of 0.5 msec was used.

Oxygenated (+ 5 % CO_2) Tyrode solution was used, and undissolved NaCl was added to the bath in an amount necessary to double the concentration of that salt. Decreased concentration of NaCl was used in a few experiments. Sucrose was added to make the solution isotonic. 1 VRU hyaluronidase (Invasin® Lundbeck") per 100 ml was used in one experiment to make the connective tissue of the preparation more permeable for the salt solution and to obtain the neuromuscular block as rapidly as possible.

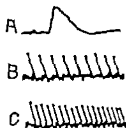


Fig. 5. E.p.p. of rat diaphragm preparation blocked by use of low sodium (appr. 1/3 of normal conc., sucrose added to compensate for loss of molarity). Recorded 20 min after change of fluid. A: single e.p.p. before tetanic volleys with 50/sec (B) and 100/sec (C).

a complete block could be obtained (with superimposed spike potential clearly distinguishable from e.p.p.) and we really found a well sustained increase of approximately 15 % of the original e.p.p. This result has, however, been omitted from Fig. 3, because movements of still contracting preparation may cause small irrelevant variation in the amplitude of e.p.p. The increase in e.p.p. is, however, *in accordance with the slow increase in the amplitude of* the contractions before complete block is obtained.

Fig. 4 demonstrates potentials obtained by stimulation with frequencies of 50 and 100/sec. shortly before pure e.p.p. was obtained. Only a very small fraction is not e.p.p., and no contraction occurs during tetanic stimulation at this stage of approximately complete block. We found a slight but readily observable increase of potentials in this introductory stage of stimulation when 50/sec. was used and an approximately stable level with 100/sec. At a later stage of the NaCl action we usually found a small decrease of the e.p.p. just after the beginning of the stimulation, and our results are in this respect in conformity with those of THESLEFF. The decrease was, however, never of the magnitude seen at the stage of curarization when a Wedensky inhibition is well developed (LILLEHEIL and NAESS 1960 and 1961).

To complete the picture the possible change in the amplitude of the e.p.p. after reduction of NaCl have also been tested (Fig. 5). The stability of the e.p.p., especially in the first stage of a tetanus was again the most remarkable feature of this type of block, even if there was a slow decrease of the e.p.p. during a long sustained tetanus.

Discussion

Both mechanical recording and registration of e.p.p. demonstrate that no Wedensky inhibition is obtained by increased concentrations of NaCl. It seems, therefore, impossible to explain the mechanism behind any Wedensky inhibition by a hypothesis based on a decline of the e.p.p. during the influence of increased amounts of these ions. The reduction of e.p.p. is not at all a con-

stant phenomenon. If a reduction takes place, it develops very slowly and it is relatively small.

An increase of the e.p.p. can be obtained even in the first phase of stimulation, when the mechanism behind the very rapidly developing Wedensky inhibition — for example during curarization — must be at work.

HUTTER (1952) also failed to find any change of the ACh-sensitivity of the motor end-plate during a Wedensky inhibition produced by tubocurarine, and concluded that this phenomenon must be of pure presynaptic origin, a result which also conflicts with the hypothesis put forward by THESLEFF (1959). (See addendum.)

Previously described changes in the neuromuscular transmission produced by increased sodium or increased osmotic pressure

Increased concentrations of NaCl probably have two different effects on the processes involved in the neuromuscular transmission: i) the specific effects of the increased concentrations of the two ions, ii) an unspecific effect of the increased osmotic pressure. FATT and KATZ (1952) examined the effect of increased concentrations of sodium and of sucrose on the e.p.p. of frog and found two opposite effects. Sucrose decreased the e.p.p. but sodium itself produced a tendency to increase. The two effects may compensate for each other keeping the e.p.p. on a steady level but the increasing effect of the sodium ion overcompensates for the depressive effect of the increased osmotic pressure when curarized preparations are used.

LILEY (1956) demonstrated a double effect of increased osmotic pressure produced by sucrose, *viz.* a significant increase of the frequency of the miniature potentials and at the same time a reduction of the e.p.p. was obtained (40 % with a 25 % increase in the osmotic pressure). An increase of the frequency of the miniature potentials is believed to be an indication of a tendency to increased release of ACh by nerve impulses (ECCLES, PIHL and LILEY 1959). Two different mechanisms which counteract each other seem, therefore, to be in action during increase of osmotic pressure, the postsynaptic decrease of the acetylcholine-sensitivity being the dominant one.

THESLEFF found no change of the amplitude of the miniature potentials in his experiments with increased NaCl-concentrations, a finding which was taken as a proof of an unchanged sensitivity of the end-plate. This may be in accordance with the results of FATT and KATZ, who have demonstrated that different mechanisms which compensate each other are at work under such circumstances. The unchanged ACh-sensitivity under the influence of increased NaCl-concentrations does, thus, not imply that the postsynaptic membrane is uninfluenced by the unphysiological condition produced by the high NaCl-concentration and reacts in a normal manner to different experi-

mental procedures — as for example — the combination of tetanic stimulation and ACh-application used by THESLEFF

Different stages of effect of increased NaCl-concentration

The neuromuscular effects of small ions such as magnesium and calcium are rapidly produced, and it is also highly probable that the specific effects of sodium are produced as rapidly while the effects which are due to the increased osmotic pressure must take some time to become fully established. It is obviously impossible to distinguish completely between these two actions in an experiment. It is, however reasonable to presume that the specific effect of sodium is the predominant one in the first phase of action, while that of the changed osmotic pressure produces its strongest effect in a later stage. When both mechanisms of action have produced their full effect the preparation works under such unphysiological conditions, that deductions about physiological behaviour must be made with the utmost caution.

Result of present investigation in relation to previous results

In the first phase of the action of the increased NaCl-concentration we found a tendency to augmentation of the e.p.p. — and no decrease — even if stimulation with frequencies as high as 100/sec was used. The decrease of e.p.p obtained during stimulation at a later stage was, furthermore, limited. The relative constancy of the amplitude of the e.p.p. during sustained tetanic stimulation was in our investigation the most remarkable feature of the NaCl-inhibited transmission. It is, therefore, not impossible that the results obtained by THESLEFF *i. e.* the strongly reduced effect of electrophoretically applied ACh immediately after stimulation with tetanic bursts of moderate frequencies, may be due to the unphysiological condition of the preparation.

The mechanism behind the low sodium block will not be discussed in this paper The reader is referred to the work of FATT and KATZ. The only point which it is necessary to keep in mind for this discussion is the stability of the e.p.p. also when the block has been obtained by this method. It is tempting to state that the consistant conclusion of our experiments is The e.p.p probably does not change even during the introductory stage of a tetanus under normal physiological conditions, when no distinct change can be observed immediately after attainment of pure measurable e.p.p. by either low or high sodium. The typical Wedensky inhibition, characterized by transformation of a tetanic contraction to a very shortlived twitch, is according to this conclusion probably not caused by any normally existing change of the output or sensitivity to ACh during the first phase of tetanus. This conclusion is also consistant with results obtained during the introductory stage of curarization, when the stability of the e.p.p. also was the relatively surprising experimental finding (LILLEHEIL and NAESS 1960 and 1961)

More direct evidence of the ACh-sensitivity of the motor end-plate has previously been obtained by LILEY (1956). This author has demonstrated that no change of the amplitude of the miniature potentials occurs during or immediately after a tetanus. This seems to be a direct proof of an unchanged sensitivity of the motor end-plate during tetanic stimulation. In this connection there is also reason to repeat the results of HUTTER, quoted in the beginning of the discussion. The acetylcholine sensitivity is not changed during a Wedensky inhibition, i. e. during a tetanic stimulation. It is still very difficult to examine e.p.p. changes during a tetanus in a mammalian preparation uninfluenced by drugs, toxins or ions. It is, however, tempting to look to the ganglionic transmission for comparison. ECCLES (1955) has recorded synaptic potentials by microelectrodes in ganglia of rabbits. The relative stability of these potentials, even at relatively higher frequencies (up to 40—50/sec) was remarkable in this investigation too. A further discussion of some of these and similar results will be taken up in our following work on the presynaptic effect of tubocurarine.

We would, however, at the end of this discussion emphasize that it is impossible by our experiments to prove that desensitization does not occur at all during a tetanus. It may, of course, be that development of desensitization is counteracted and compensated — or even overcompensated by a real increase of the release of ACh during the first phase of a tetanic stimulation. It may be that such a relatively small naturally occurring desensitization is aggravated under the conditions brought about by the increased NaCl-concentration and the hyperosmolality produced thereby. THESLEFF's results demonstrate quite clearly that a desensitization really exists under the experimental conditions of his experiments. The decrease of the e.p.p. produced by administration of ACh was, however, surprisingly strong compared with the often relatively small decline in the e.p.p. produced by indirect stimulation. This seems to imply that an increase in the ACh-release per impulse probable must take place during the introductory phase of tetanic stimulation. Other reports which will not be mentioned here, indicate that the activity in the *presynaptic fibres combined with conduction or electronic spread* of the action potential and the release of ACh produced thereby, is followed by a state of increased preparedness for releasing ACh (for references see ECCLES *et al.* 1959). Such a mechanism would produce a potentiation of ACh-release during the transmission of repetitive stimuli — a process eventually compensating for a possible desensitization. More indirect evidence seems for the present — as mentioned above — to contradict a process of desensitization during a short tetanus under physiological conditions. That a desensitization to ACh takes place during a more prolonged tetanic contraction has been demonstrated by KRNJEVIC and MILEDI (1958). The course of the release of ACh during tetanic stimulation will be more comprehensively discussed in our following paper on the presynaptic effect of tubocurarine.

Addendum

Otsuka and Endo (1960) have quite recently published results obtained in experiments with frogs to test the hypothesis of desensitization put forward by Thesleff. They used intracellular recording of e.p.p. and electrophoretical application of ACh in curarized preparations. No sign of desensitization to ACh could be recorded after conditioning repetitive stimulation. Otsuka and Endo, therefore concluded that the sensitivity of the end-plate to ACh remains entirely unchanged even when the amplitude of end-plate potential is markedly decreased after repetitive stimulation. Their conclusion was, accordingly in agreement with that of Hutter (1952) quoted in our article: The Wedensky inhibition must be due to a pure presynaptic procedure. Otsuka and Endo mention that the difference between their results and conclusion and Thesleff' might be due to use of different species, but Hutter used mammalian preparation.

References

Bülbring, E., Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Brit. J. Pharmacol.* 1946. *1* 38—61.

Eccles, R. M., Intracellular potentials recorded from mammalian sympathetic ganglion. *J. Physiol.* (Lond.) 1955 *130* 572—584.

Eccles, J. C., D. Pillar and A. W. Liley Factors controlling the liberation of acetylcholine at the neuromuscular junction. *Amer. J. Phys. Med.* 1959 *38*, 96—105

Fatt, P. and B. Katz, The effect of sodium in the neuromuscular transmission. J. Physiol. (Lond.) 1952. *118*. 73—87

Howarth, J. V. The behaviour of frog muscle in hypertonic solutions. *J. Physiol.* (Lond.) 1958. *144* 167—175.

Hutter, O. F., Post-tetanic restoration of neuromuscular transmission blocked by d-tubocurarine. *J. Physiol.* (Lond.) 1952. *118*. 216—227

Krnjević, K. and R. Miledi, Failure of neuromuscular propagation in rats. *J. Physiol.* (Lond.) 1958. *140*. 440—461

Liley, A. W. An investigation of spontaneous activity at the neuromuscular junction of the rat. *J. Physiol.* (Lond.) 1956. *132*. 650—666.

Lilleheil, G. and K. Naess, Presynaptic effect of d-tubocurarine. Experientia. 1960. *16* 550—551

Lilleheil, G. and K. Naess, Presynaptic effect of d-tubocurarine. Acta physiol. Scand. In Press.

Otsuka, M. and M. Endo, Presynaptic nature of neuromuscular depression in frog. Nature 1960. *188* 501—502.

Thesleff, S., Motor end-plate "desensitization" by repetitive nerve stimuli. *J. Physiol.* (Lond.) 1959 *148*. 659—664.

Acta physiol. scand. 1961. 52. 32—35

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Respiratory Response to Acute Exercise in Induced Metabolic Acidosis

By

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Abstract

Refsum, H. E. *Respiratory response to acute exercise in induced metabolic acidosis.* Acta physiol. scand. 1961. 52. 32—35. — Ventilation, carbon dioxide output and oxygen uptake were recorded before, during and after a work of 500 kgm in one minute — before and after inducing a marked metabolic acidosis by means of ammonium chloride. While the resting ventilation in the acidotic state was moderately increased, the ventilatory increase due to the exercise was extremely high. The excess carbon dioxide blown off simultaneously however was very moderate. The increases of the resting and working ventilations were due to increases in the tidal volumes only. The recovery times of carbon dioxide output and ventilation were markedly increased, while the recovery times of oxygen uptake showed no definite change. The described pattern of respiratory response to acute exercise is often found in patients with cardiac or cardiopulmonary disease.

Using a standard exercise test for evaluation of cardiorespiratory function (Eriksson 1952, 1957) consisting of recording carbon dioxide output, oxygen uptake and ventilation in connection with the performance of 500 kgm work in one minute, Lange Andersen (1960) has found carbon dioxide recovery times of 2—5 min and ventilatory increases of 20—40 l (ATPS) in young, healthy men. Eriksson (1960) has found that in severe cardiorespiratory disease the carbon dioxide recovery times may be increased up to 15—20 min, and Eriksson and Müller (1960) have shown that abnormally high ventilatory in-

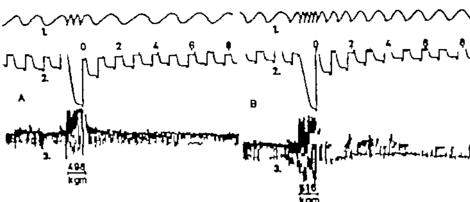


Fig. 1 Sections of the spirometer curves, recorded before intake of ammonium chloride (A) and after intake of 55 g ammonium chloride in the course of 48 hours (B). (1 Ventilation. 2: Carbon dioxide output. 3: Respiratory difference curve.)

creases, exceeding 60 l (ATPS) in patients with pulmonary disease, and 45 l in patients with no signs of pulmonary disease, strongly indicate cardiac disease, provided that no acidosis is present.

Earlier investigations have demonstrated the influence of experimentally induced metabolic acidosis on the ventilation at rest and during steady state work (Haldane 1921 Dennig *et al.* 1931 Nielsen 1936, and others) The purpose of this work has been to investigate the influence of induced metabolic acidosis on the respiratory response to acute exercise.

Methods and procedure

The standard exercise test was performed according to Erikson (1952, 1957) by re cording the carbon dioxide output, oxygen uptake and pulmonary ventilation per minute during resting period of 20 min, one minute exercise (performing 500 kgm on bicycle ergometer) and the following 20 min of recovery and rest.

The recovery times for the carbon dioxide output, oxygen uptake and ventilation were defined as the times from the end of the exercise till the value of the actual parameter was less than 10 per cent bove the mean resting value. The increases in carbon dioxide output, oxygen uptake and ventilation due to the exercise were determined as the dif ferences between the total value of the actual parameter during 10 min period — including the last pre-exercise minute, the exercise minute and the following 8 min — and the mean resting values for 10 min.

The exercise test was performed on 3 consecutive mornings by a healthy well trained male subject (32 years, height 190 cm, weight 79.0 kg). Between the first and the second test 10 portions of 2.5 g ammonium chloride were taken *per os* between the second and the third test additionally 30 g were taken in the same way The total intake of ammonium chloride in the course of 48 hours was thus 55 g.

Before each exercise test venous blood sample was taken, and the CO combining power of plasma determined according to Van Slyke and Cullen (1917) Forty-five minutes after the last test an arterial blood sample was taken and the blood gas values deter mined (Rerrum 1960)

Table I. Respiratory response to acute exercise in induced metabolic acidosis. (Control: Before intake of ammonium chloride. 24 hrs: After intake of 25 g ammonium chloride in the course of 24 hours. 48 hrs: After intake of additional 30 g ammonium chloride in the following 24 hours.)

	Control	24 hrs.	48 hrs.
Resting oxygen uptake, ml/min	263	274	276
carbon dioxide output, ml/min	238	246	242
ventilation, l/min	7.9	9 4	10.5
respiratory rate	13.7	13.3	15.8
Work, kgm performed in one min	498	510	516
Net work efficiency per cen	20 4	20 8	19.7
Increase in ventilation due to work, l	22.5	44 4	64 0
Working respiratory rate	16	16	17
Increase in carbon dioxide output due t work, l	1.25	1.35	1 63
Recovery time of carbon dioxide output, min	1	3	4
oxygen uptake min	1	2	2
» entilation, min	1	3	5

All volumes are given at ATPS.

Results

On the first experimental day the CO combining power of plasma was found to be 26 0 meq/l (i e quite normal) while it before the second test (after intake of 25 g ammonium chloride in the course of 24 hours) had decreased to 15.7 meq/l, and before the third exercise test (after an additional intake of 30 g during the following 24 hours) it was 13 5 meq/l An arterial blood sample taken 45 min after the last exercise test showed CO content (plasma) 11.5 meq/l, pH 7 13 CO tension 32 mm Hg, hemoglobin oxygen saturation 97 5 per cent. During the experiment the body weight changed from 79 0 kg on the first day to 77 0 kg on the second, and 76 0 kg on the third day

Fig 1 shows sections of the spirometer curves, recorded on the first day before the intake of ammonium chloride, and on the third day after intake of 55 g in the course of 48 hours. The data calculated from the three curves recorded are presented in Table I

Comments

The resting oxygen uptake and carbon dioxide output, the work performed and the net work efficiency varied within relatively narrow limits in all tests. This indicates that the experimental conditions as far as the exercise tests are concerned must have been practically identical.

The intake of ammonium chloride led to a marked increase of the resting ventilation, the last value being 33 per cent higher than the control value but all values were within the range for normal subjects. The ventilatory in-

crease due to the exercise increased from a low normal value in the control test to a high, normal value on the first day with acidosis, and an abnormally high value on the second day with severe acidosis this value being approximately 185 per cent higher than the control value. It appears further that the respiratory rates, both for the resting periods and the exercise minutes, were the same in all three tests, showing that the increases in resting and working ventilation were the results of increases in the tidal volumes only.

While the resting values of carbon dioxide output were practically the same in all three tests, the increase in carbon dioxide output due to the exercise increased clearly during the acidosis, the last value being approximately 30 per cent higher than the control value. It appears, however that the very high increases in the ventilation due to the exercise only led to a very moderate excess output of carbon dioxide, and consequently to a very low carbon dioxide concentration in the expired air (cp ERIKSON 1957). The ventilation and carbon dioxide recovery times increased from low normal values in the control test to high, normal values in the last test, while the oxygen recovery time showed no definite change.

It is seen that experimentally induced metabolic acidosis in a normal subject, without signs of cardiopulmonary or cardiac disease, can lead to the same respiratory response to brief, relatively moderate exercise as often found in patients with definite cardiac or cardiopulmonary disease (ERIKSON and MÜLLER 1960) i. e. with resting ventilation within the normal range, and abnormally high ventilatory increase due to the exercise, and increased carbon dioxide recovery time.

References

DENNIG, H., J. H. TALBOTT, H. T. EDWARDS and D. B. DILL, Effect of acidosis and alkalosis upon capacity for work. *J. clin. Invest.* 1931, *9*, 601—613.

ERIKSON, H., Spirometry during short exercise test for evaluation of pulmonary function. *Scand. J. clin. lab. Invest.* 1952, *4*, 336—344.

ERIKSON, H., The respiratory response to acute exercise of eskimoes and white. *Acta physiol. scand.* 1957, *41*, 1—11.

ERIKSON, H., Unpublished data. 1960.

ERIKSON, H. and O. MÜLLER, Cardiopulmonary disease and pulmonary ventilation. *Acta chir. scand.* 1960. Suppl. 253, 111—117.

HALDANE, J. B. S., Experiments on the regulation of the blood's alkalinity II. *J. Physiol. (Lond.)* 1921, 55, 265—275.

LANGE ANDERSEN, K., Respiratory recovery from muscular exercise of short duration. *Acta physiol. scand.* 1960, *48*, Suppl. 168.

NIELSEN, M. Untersuchungen über die Atemregulation beim Menschen. *Skand. Arch. Physiol.* 1936, *74*, Suppl. 10, 87—208.

REFSUM, H. E., Arterial blood gases in respiratory insufficiency. *Acta chir. scand.* 1960. Suppl. 253, 175—181.

VAN SLYKE, D. D. and G. CULLEN, Studies of acidosis. I. The bicarbonate concentration of the blood plasma, its significance, and its determination as measure of acidosis. *J. biol. Chem.* 1917, 30, 289—318.

Acta physiol. scand. 1961 52. 36—43

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Effect of Dietary Selenium Dioxide, Cystine, Ethoxyquin and Vitamin E on Lipid Autoxidation in Chick Tissues

By

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Abstract

BIERI, J G H. DAM, I PRANGE and E. SONDERGAARD, *Effect of dietary selenium dioxide, cystine ethoxyquin and vitamin E on lipid autoxidation in chick tissues.* Acta physiol. scand. 1961 *52* 36—43. — The ability of dietary selenium dioxide, L-cystine, ethoxyquin (1,2-dihydro-6-ethoxy 2,2,4-trimethylquinoline) and dl-α-tocopheryl acetate to inhibit autoxidation in incubated tissue homogenates was studied using the thiobarbituric acid test. Ethoxyquin (0.1 %) was as effective a tissue antioxidant as was dl-α tocopheryl acetate (0.01) SeO_2 (0.14 0.46 and 1 4 ppm) significantly inhibited autoxidation in the liver kidney and heart the effect varied with different vitamin E-free diets. L-cystine (0.3 %) reduced autoxidation in muscle when fed in a casein-gelatine diet.

The autoxidation of tissue fatty acids which occurs when homogenates or cell particulate components are incubated in air can be greatly reduced if sufficient antioxidant, in the form of vitamin E, is fed to animals prior to the removal of tissues (TAPPEL and ZALKIN 1959, MACHLIN et al. 1959 BIERI and ANDERSON 1960) In addition to this effect by vitamin E, it was found that under

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Table I Composition of basal diets in percentages

	Diet no.		
	2,304	2,343	2,303
Torula yeast 3N	60		
Soybean protein		30	
Casein			15
Gelatine	5		10
Salt mixture	5.17	5.17	5.17
Vitamin mixture	0 1	0.1	0.1
Choline chloride	0.2	0.2	0.2
Sucrose	31.53	63.33	68.33
Lard		1	1

The diets were supplemented with 1 mg Synkavit "Roche (Di-calcium salt of 2-methyl-1 4-naphthohydroquinone-diphosphoric acid ester) per 100 g. Vitamins A and D_3 were given as solution prepared from crystalline vitamin A acetate (Roche) 1 g crystalline vitamin D (Roche), 0.0058 g "Tween 80, 64 g ethyl alcohol, 100 ml; and distilled water t total volume of 330 ml. 0.1 ml twice week per animal furnished 250 I. u. vitamin A and 20 I. u. vitamin D_3 per day

From Lake States Yeast Corporation, Rhinelander Wisconsin, U S A.
ADM Assay Protein C-1 from Archer Daniels-Midland Company Cincinnati, Ohio, U S. A.
"Dabrinex, from A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark.
Dam and Søndergaard (1953)

some conditions the feeding of selenite to chicks significantly decreased lipid peroxidation in liver while the feeding of L-cystine decreased peroxidation in muscle (BIERI 1959) The present report extends these observations to other tissues when chicks are fed different types of vitamin E-free diets.

Experimental

Day-old New Hampshire and Whit Leghorn chicks were given a commercial starter ration for 6 days. They were then grouped and fed the experimental diets shown in Table I The vitamin E-free basal diets differed primarily in their protein source. After 21 to 35 days on the experimental diets, the birds were sacrificed and the tissues removed. Testing of autoxidation of fatty acids was begun the day of sacrifice; however for some tissues storage t — 20° C for period up to 74 days was required to finish all the determinations. W have observed (unpublished results) that frozen storage of tissues may reduce the degree of lipid autoxidation which occurs upon incubation. In this study however similar tissues from all groups within one experiment were analyzed t the same time.

The tissues were homogenized and incubated as described previously (BIERI and ANDERSON 1960) Briefly 3 to 5 ml of a 5 % homogenate in phosphate buffer pH 7 4 was pipetted into 50 ml Erlenmeyer flasks. These were incubated for one hour in 37° C water bath, with shaking, in an atmosphere of air Thereafter 1 ml aliquots were pipetted

Table II Summary of the incidences of vitamin E deficiency symptoms

Group no.	Basal diet no.	Source of protein	Addition to basal diet	Days on diet	Incidence of		
					Exudative diathesis	Encephalomalacia	White striation of breast muscles
2,301	2,301	Torula yeast, gelatine	None	22 – 32	9^{10}	1^{10}	
2,315			1.4 ppm SeO		0^{9}	0^{9}	
2,316			0.46 ppm SeO		0^{9}	1^{9}	
2,317			0.14 ppm SeO_2		0^{9}	1^{9}	
2,308			0.1 Ethoxyquin		1^{10}	0^{10}	
2,311			0.01 " dl-α Tocopheryl acetate		0^{9}	0^{9}	
2,343	2,343	Soybean-protein	None	21 – 33	15^{31}	0^{31}	16^{31}
2,344			0.3 " L-Cystine		8^{19}	0^{19}	0^{19}
2,345			0.1 Ethoxyquin		0^{19}	0^{19}	0^{19}
2,346			0.46 ppm SeO		0^{19}	0^{19}	0^{19}
2,347			0.01 " dl-α Tocopheryl acetate		0^{19}	0^{19}	0^{19}
2,383	2,383	Casein, gelatine	None	31 – 35	0^{10}	0^{10}	8^{10}
2,384			0.3 " L-Cystine		0^{10}	0^{1}	1^{10}
2,387			1.4 ppm SeO		0^{10}	0^{10}	3^{10}
2,389			0.01 dl-α Tocopheryl acetate		0^{10}	0^{10}	0^{10}

The superscripts indicate the number of chicks in the group.

Table III Thiobarbituric acid (TBA) values in homogenates of various tissues from chicks fed diets containing 60 % Torula yeast. Feeding period 22 to 32 days

Group	Addition to basal diet no. 2,301	No of chicks	Mean TBA values ± standard error				
			Liver	Kidney	Spleen	Breast muscle	Lungs
2,301	None	7	356 ± 50	150 ± 23	223 ± 33	33 ± 13	24 ± 13
2,315	1.4 ppm SeO	7	129 ± 14	117 ± 14	230 ± 12	36 ± 4	122 ± 9
2,316	0.46 ppm SeO	7	111 ± 15	112 ± 6	226 ± 12	39 ± 4	155 ± 11
2,317	0.14 ppm SeO	7	101 ± 16	129 ± 9	201 ± 10	45 ± 8	136 ± 8
2,308	0.1 % Ethoxyquin	7	19 ± 6	8 ± 1	31 ± 7	5 ± 1	11 ± 1
2,311	0.01 % dl-α Tocopheryl acetate	7	20 ± 5	65 ± 14	22 ± 2	6 ± 2	13 ± 1

Absorbancy at 530 mμ × 1,000 ± standard error
TBA test performed on the day of sacrifice
TBA values estimated on tissues stored 6 to 15 days at −20° C.
1.4 ppm SeO_2 = 1 ppm Se.

into 1.5 ml of 10 % trichloracetic acid. The extent of autoxidation of unsaturated fatty acids was estimated on the deproteinized solution as described previously using thiobarbituric acid (BIERI and ANDERSON 1960). The pink colors produced by the reaction of the reagent with malonic dialdehyde, an end-product of lipid peroxidation, were measured in a Beckman model C colorimeter. A reagent blank was carried through the color development procedure. The intensity of the color and hence the amount of autoxidation, is reported as thiobarbituric acid value (TBA value) which is the absorbancy reading at 530 mμ × 1 000.

Differences in TBA values between group means are considered significant only if the analysis of variance indicated probability of less than 0.01.

Results

A high incidence of vitamin E-deficiency symptoms occurred in chicks fed each of the basal diets (cf. Table II). Thus, the Torula yeast and soybean protein diets produced a high incidence of exudative diathesis. The additions of selenium dioxide, ethoxyquin or dl-α-tocopheryl acetate counteracted the exudates. The casein and soybean protein diets produced white muscle striations (DAM, PRANGE and SONDERGAARD 1952). This latter symptom was counteracted by L-cystine, selenium dioxide, ethoxyquin and dl-α-tocopheryl acetate.

As reported previously (BIERI 1959, BIERI and ANDERSON 1960), the autoxidation in brain was higher than in any other tissue studied and was also completely unaffected by any dietary supplement. Consequently, the results with brain are not included and will not be considered when comparisons are made between other tissues.

The TBA values obtained from tissues of chicks fed three different levels of SeO_2 and also the antioxidant ethoxyquin, in the Torula yeast diet are shown in Table III. 0.1 % ethoxyquin was fully as effective as 0.01 % dl-α-tocopheryl acetate in preventing autoxidation in all tissues and may have been even more effective than vitamin E in the kidney. SeO_2 (0.14, 0.46 and 1.4 ppm), however, exerted an antioxidant action only in the liver but the effect was not as great as that of 0.01 % dl-α tocopheryl acetate or 0.1 % ethoxyquin. The TBA values, which were similar for all three levels of SeO_2, represent a very pronounced decrease in autoxidation compared with the control, unsupplemented group.

An unexplained phenomenon was found in the lungs, where SeO_2 increased the autoxidation over that of the control group. This was not observed in succeeding experiments (below) with other diets.

In Table IV are shown the results obtained when L-cystine, ethoxyquin and SeO_2 were incorporated into the soybean protein diet. In this experiment (and also in the following one), in addition to incubating the homogenates alone an aliquot was also incubated with 0.1 μmole ascorbic acid per ml homogenate. Ascorbate has been shown to increase the autoxidation of fatty acids in tissues (OTTOLENGHI 1959). By incubating the homogenates both with

1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline ("Santoquin"), Monsanto Chemical Co., St. Louis, Mo., U.S.A.

Table IV Thiobarbituric acid (TBA) values in homogenates of tissues from chicks fed diets

Group no.	Addition to basal diet no. 2,343	Number of chicks	Mean values ± standard error			
			Liver		Kidney	
			−C	+C	−C	+C
2,343	None	16	104 ± 14	140 ± 25	131 ± 8	238 ± 15
2,344	0.3 % L-Cystine	9	96 ± 29	112 ± 36	118 ± 11	231 ± 20
2,345	0.1 % Ethoxyquin	9	28 ± 3	32 ± 6	12 ± 2	13 ± 2
2,346	0.46 ppm SeO	10	37 ± 6	37 ± 7	69 ± 10	123 ± 22
2,347	0.01 % dl-α Tocopheryl acetate	10	22 ± 2	20 ± 2	37 ± 5	47 ± 6

Absorbancy at 530 mμ × 1,000 ± standard error
Tissues were stored up to 74 days at −20° C before estimation of TBA values.
TBA tests were performed on the day of sacrifice. Determinations with added ascorbic −C = no added ascorbic acid; +C = 0.1 μmole ascorbic acid added per ml homogenate.
0.46 ppm SeO_2 = 0.33 ppm Se

and without ascorbic acid it is possible to show that observed differences between the various groups are not due simply to differences in ascorbic acid content of the tissues (Bieri and Andersson 1960)

The most striking results in this experiment were those obtained with tissues from SeO_2-fed chicks. The antioxidant effect of SeO on the liver was considerably more pronounced with the soybean protein diet than with the Torula yeast diet. In fact, the almost complete inhibition of autoxidation in the liver by SeO was similar to that produced by ethoxyquin, and almost as complete as that produced by dl-α-tocopheryl acetate.

Table V TBA values in homogenates of tissue from chicks fed diets containing 15 % casein and

Group no.	Addition to basal diet no. 2,583	Number of chicks	Mean TBA values ± standard error			
			Liver		Kidney	
			−C	+C	−C	+C
2,583	None	10	41 ± 5	53 ± 6	58 ± 2	114 ± 8
2,584	0.3 L-Cystine	8	47 ± 6	59 ± 9	58 ± 4	98 ± 9
2,587	1.4 ppm SeO_2	10	52 ± 3	67 ± 3	37 ± 5	129 ± 12
2,589	0.01 dl-α Tocopheryl acetat	9	18 ± 1	20 ± 1	24 ± 3	30 ± 3

Absorbancy at 530 mμ × 1,000 ± standard error
−C = no added ascorbic acid, +C = 0.1 μmole ascorbic acid added per ml homogenate
1.4 ppm SeO_2 = 1 ppm Se.
The tissues were stored up to 24 days at −20° C before estimation of TBA values.

containing 30 % soybean protein. Feeding period 21 to 33 days

Mean values ± standard error

Breast muscle		Heart		Lungs		Spleen
—C	+C	—C	+C	—C	+C	—C
44 ± 5	153 ± 8	78 ± 6	273 ± 12	37 ± 5	58 ± 7	149 ± 8
50 ± 6	73 ± 11	86 ± 9	287 ± 11	54 ± 8	71 ± 7	152 ± 10
36 ± 6	57 ± 10	19 ± 3	21 ± 3	29 ± 5	34 ± 6	89 ± 9
34 ± 6	68 ± 11	57 ± 5	216 ± 19	40 ± 5	48 ± 6	112 ± 15
32 ± 6	38 ± 7	14 ± 3	14 ± 2	13 ± 3	16 ± 3	11 ± 2

acid were not made.

A significant decrease in TBA values as a result of feeding SeO was also noted in the kidney and heart both in the absence and presence of ascorbic acid.

In the third experiment, diet no. 2383 containing 15 % of casein, 10 % of gelatine and 1 % of lard (Table I) was used. This diet produced white muscle striation in 9 out of the 10 chicks (Table II) The TBA values of tissues from chicks fed this diet with cystine, SeO or vitamin E are shown in Tabl V Both cystine and SeO produced a significant inhibition of lipid autoxidation in muscle. In addition, SeO reduced the TBA value for heart but only in the absence of ascorbic acid.

10 % gelatine. Feeding period 34 to 35 days

Mean TBA values ± standard error

Breast muscle		Heart		Spleen	Lungs	
—C	+C	—C	+C	—C	—C	+C
37 ± 3	106 ± 8	65 ± 5	225 ± 10	101 ± 12	44 ± 3	61 ± 4
19 ± 1	68 ± 5	52 ± 3	214 ± 10	65 ± 11	58 ± 3	55 ± 5
23 ± 2	80 ± 5	45 ± 5	244 ± 12	129 ± 10	40 ± 2	67 ± 4
7 ± 1	7 ± 1	12 ± 1	14 ± 2	31 ± 3	11 ± 1	14 ± 1

Discussion

These results confirm and extend the previous observations (BIERI 1959) of the antioxidant effect of dietary selenite and L-cystine on certain tissues. In addition to the effect on the liver found before, it is apparent that under appropriate dietary conditions, as with the soybean protein diet, SeO_3 also inhibits autoxidation in other tissues, specifically the heart and kidney. This action of SeO_3, however, is strongest in the liver but the results suggest that perhaps the action is widespread in the body. The observation in the experiment with Torula yeast that three different dietary levels of SeO_3 all inhibited autoxidation in the liver to the same degree suggests that the tissue concentration of biologically active selenium is not increased by higher supplements of the element.

It should be pointed out that the Torula yeast and soybean protein are deficient in biologically active selenium, since they can be used to produce exudative diathesis. The casein, however, contains sufficient selenium to prevent exudates with the level of lard used in this experiment.

The total selenium content of the various proteins determined by neutron activation analysis was for Torula yeast 0.038 μg, for casein 0.382 μg, for soybean protein 0.14 μg, and for gelatine 0.088 μg selenium per gram substance (SCHWARZ 1960).

Further, it should be emphasized that the Torula yeast contains 2.4 % dienoic and 0.14 % trienoic fatty acids (DAM et al. 1957), the soybean protein contains 0.65 % dienoic and 0.07 % trienoic, whereas casein is practically devoid of polyenoic fatty acids.

In the case of L-cystine, its antioxidant effect seems restricted to the muscle. The restriction of the effect to muscle is in accord with the nutritional role of L-cystine in chicks in that the only vitamin E-deficiency symptom it will prevent is white muscle striation.

The effectiveness of the antioxidant ethoxyquin in inhibiting autoxidation in all tissues (except brain) agrees with the observation that this compound at least in some respects will replace vitamin E in the chick (MACHLIN *et al.* 1959, SØNDERGAARD et al. 1960).

Although we do not know the precise mechanism whereby dietary SeO_3 and L-cystine exert their antioxidant action in tissues, there is sufficient evidence to permit some hypotheses to be made. Since it is known (BIERI 1959) that selenite and L-cystine added to tissue homogenates will not inhibit lipid autoxidation, it is apparent that these substances when fed either alter the normal chemical composition of the cell or give rise to new or modified compounds which act as antioxidants. Since cystine and selenium are incorporated into cell proteins, the latter postulate seems more feasible although the two probably have different sites of action. In the case of selenium, it may be that the substitution of selenium for sulfur in the proteins, particularly in the liver, yields a new protein with remarkable antioxidant properties.

We are indebted to Dr Klaus Schwarz, National Institutes of Health, Bethesda, Md., U. S. A., for the neutron activation analysis of the proteins for trace selenium, to Lake States Yeast Corporation, Rhinelander, Wisconsin, U. S. A., for the supply of Torula yeast, and to F. Hoffmann-La Roche & Co., Ltd., Basle, Switzerland, for having provided the "Synkavit" and dl-α-tocopheryl acetate ("Ephynal") used in these studies.

References

Bieri, J. G. An effect of selenium and cystine on lipide peroxidation in tissues deficient in vitamin E. *Nature* (Lond.) 1959. *184* 1148—1149.

Bieri, J. G. and A. A. Anderson, Peroxidation of lipids in tissue homogenates as related to vitamin E. *Arch. Biochem. Biophys.* 1960. *90* 105—110.

Dam, H., I. Prange and E. Søndergaard, Muscular degeneration (white striation of muscles) in chicks reared on vitamin E deficient, low fat diets. *Acta path. microbiol. scand.* 1952. *31* 172—184.

Dam, H. and E. Søndergaard, Comparison of the action of vitamin K_1, menadione and Synkavit administered intravenously to vitamin K deficient chicks. *Acta pharmacol.* (Kbh.) 1953. *9* 131—136.

Dam, H., G. K. Nielsen, I. Prange and E. Søndergaard, The exudative diathesis produced by Torula yeast. *Experientia* (Basel) 1957 *13* 493.

Machlin, L. J., R. S. Gordon and K. H. Meisky The effect of antioxidants on vitamin E deficiency symptoms and production of liver "peroxide" in the chicken. *J. Nutr.* 1959. *67* 333—343.

Ottolenghi, A. Interaction of ascorbic acid and mitochondrial lipides. *Arch. Biochem. Biophys.* 1959. *79* 355—363.

Schwarz, K., 1960 Personal communication.

Søndergaard, E., J. G. Bieri and H. Dam, Further observations on the effects of selenium and antioxidants on exudative diathesis in chicks. *Experientia* (Basel) 1960. *16* 554—556.

Tappel, A. L. and H. Zalkin, Inhibition of lipide peroxidation in mitochondria by vitamin E. *Arch. Biochem. Biophys.* 1959 *80* 333—336.

Acta physiol. scand. 1961 52, 44—48

From the Departments of Pharmacology Universities of Lund and Göteborg, Sweden

Effect of Reserpine on the Storage of New-formed Catecholamines in the Adrenal Medulla

By

Åke Bertler, Nils-Åke Hillarp and Evald Rosengren

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Abstract

Bertler, Å., N.-Å. Hillarp and E. Rosengren. *Effect of reserpine on the storage of new-formed catecholamines in the adrenal medulla.* Acta physiol. scand. 1961 52 44—48. — When L-dopa (100 mg/kg body wt) was given i.v. to reserpinized (5 mg/kg) rabbits, appreciable amounts of dopamine and noradrenaline rapidly formed in the adrenal medulla. The results support the view that reserpine does not — at least not to a great extent — interfere directly with the decarboxylating and β-hydroxylating steps in the amine synthesis. To great extent, however the drug prevented the new-formed amines from being incorporated in the storage granules, an incorporation which in normal animals is a very rapid and efficient process. It seems reasonable to assume that reserpin in some way blocks the process of monoamine storage. This may well be the main site of action of the drug

Reserpine causes a general depletion of the monoamine stores in the body Mainly on the basis of experiments with blood platelets it has been suggested that the primary effect of this drug on 5-hydroxytryptamine (5-HT) is on the storage mechanism (*cf* Shore *et al.* 1957) It has, however hitherto been difficult to test this theory by experiments *in vivo*

A new method of studying the amine storage problem has recently become available when it was shown that large amounts of noradrenaline (NA) and dopamine (DA) are rapidly formed and stored in the adrenaline (A) cells of the rabbit suprarenal medulla after an intravenous injection of L-3,4-dihydroxyphenylalanine (dopa) (Bertler, Rosengren and Rosengren 1960, Bertler, Hillarp and Rosengren 1960 a, b) In the present paper it is shown that reserpine interferes with the storage of amines formed in this way

Material and methods

An i.v. injection of reserpine ("Serpasil" Ciba, 5 mg/kg body wt.) was given to rabbits (1.5 to 2 kg body wt.) After various periods of time L-dopa (100 mg/kg) was injected i.v. and the animals were killed 30 min to 2 hrs later by an i.v. injection of air. The adrenal medullas from each animal were homogenized in 0.3 M sucrose and centrifuged as described previously (BERTLER, HILLARP and ROSENGREN 1960). Catecholamines were determined spectrophoto-fluorimetrically (BERTLER, CARLSSON and ROSENGREN 1958, CARLSSON and WALDECK 1958.)

In 4 animals the spinal cord was transected at C6 (Nembutal narcosis) immediately before the administration of dopa.

Results and discussion

When dopa was administered to rabbits 3 to 48 hours after an i.v. injection of reserpine, appreciable amounts of NA and DA rapidly formed in the adrenal medulla (Table I). The amounts found, however, are generally lower than those found in normal animals injected with dopa (see BERTLER, HILLARP and ROSENGREN 1960 a). This could mean that reserpine in some way interferes with the amine synthesis. It has been shown, however, that denervation of the rabbit adrenal medulla largely prevents the amine depletion caused by reserpine (KRONEBERG and SCHÜMANN 1957). It is thus probable that the secretory nerves of the medulla are continuously operating in a reserpinized animal. Consequently, unknown amounts of amines formed after a dopa injection may be lost by secretion. This is in good agreement with the finding that larger amounts of NA were present in the two animals in which a transection of the spinal cord at C6 was made immediately before the dopa injection (Tabl I).

Thus the results support the view that reserpine does not — at least not to a great extent — interfere directly with the decarboxylating and β-hydroxylating steps in the amine synthesis. This is not unexpected since it has been shown that 5-HT forms in reserpinized animals in an apparently normal way (SHORE, SILVER and BRODIE 1955). There is a possibility, however, that reserpine may indirectly disturb the formation of NA and A. It has been suggested that the β-hydroxylation of DA occurs in the storage granules (KIRSHNER 1959). If this is true, reserpine may well depress this reaction to some extent since it inhibits the uptake of DA in the granules (see below).

The amines formed from injected dopa in normal rabbits are taken up and stored very rapidly by the storage granules. In reserpinized animals, on the contrary, the new-formed DA and NA were mainly recovered "free" in the cytoplasmic sap (Table I). Thus to a large extent the drug prevents the amines from being incorporated in the storage granules. This inhibition of the storage process seems to be far from complete even 24 hours after the injection of reserpine since most of the NA is particle-bound 2 hours after the dopa administration. The values actually found are probably misleading,

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Received 17 January 1961

Abstract

BERTLER, Å., N.-Å. HILLARP and E. ROSENGREN. *Effect of reserpine on the storage of new-formed catecholamines in the adrenal medulla.* Acta physiol. scand. 1961. 52. 44—48. — When L-dopa (100 mg/kg body wt.) was given i.v. to reserpinized (5 mg/kg) rabbits, appreciable amounts of dopamine and noradrenaline rapidly formed in the adrenal medulla. The results support the view that reserpine does not — at least not to a great extent — interfere directly with the decarboxylating and β-hydroxylating steps in the amine synthesis. To a great extent, however, the drug prevented the new-formed amines from being incorporated in the storage granules, an incorporation which in normal animals is a very rapid and efficient process. It seems reasonable to assume that reserpine in some way blocks the process of monoamine storage. This may well be the main site of action of the drug.

Reserpine causes a general depletion of the monoamine stores in the body. Mainly on the basis of experiments with blood platelets it has been suggested that the primary effect of this drug on 5-hydroxytryptamine (5-HT) is on the storage mechanism (*cf.* SHORE *et al.* 1957). It has, however, hitherto been difficult to test this theory by experiments *in vivo*.

A new method of studying the amine storage problem has recently become available when it was shown that large amounts of noradrenaline (NA) and dopamine (DA) are rapidly formed and stored in the adrenaline (A) cells of the rabbit suprarenal medulla after an intravenous injection of L-3,4-dihydroxyphenylalanine (dopa) (BERTLER, ROSENGREN and ROSENGREN 1960, BERTLER, HILLARP and ROSENGREN 1960 a, b). In the present paper it is shown that reserpine interferes with the storage of amines formed in this way.

medulla of reserpinized (5 mg/kg) rabbits after administration of L-dopa (100 mg/kg) parentheses)

Noradrenaline		Dopamine	
Total µg	Free Per cent	Total µg	Free Per cent
0.7 (0.5–0.9)	100	1.7 (1.4–1.9)	43 (42–44)
2.0 (1.2–2.8)	60 (54–65)	2.0 (1.4–2.5)	53 (49–57)
1.8 (1.3–2.4)	62 (53–78)	3.2 (1.8–3.8)	78 (73–82)
4.5 (3.0–5.3)	40 (21–50)	1.3 (0.8–1.6)	45 (21–60)
2.8 (1.4–4.2)	22 (14–30)	1.0 (0.7–1.3)	31 (29–33)
1.9 (1.6–2.2)	28 (21–35)	0.7 (0.6–0.8)	26 (25–27)
2.0 (0.3–3.7)	74 (63–100)	1.8 (0.3–4.5)	60 (43–89)
4.8 (3.6–6.0)	67 (65–69)	1.4 (1.2–1.7)	56 (50–62)
5.5 (3.5–7.5)	13 (12–15)	2.5 (1.8–3.2)	5 (3–7)
1.9 (1.1–2.7)	37 (23–71)	1.3 (0.2–2.4)	67 (20–92)
0.1 (0.0–0.3)	10 (0–20)	0.15 (0.1–0.2)	50 (50–50)
1.3	25		
1.7	15		
3.5	21		
0.9	13		

(1957) and HUGHES, SHORE and BRODIE (1958) who showed that reserpine prevents the uptake of 5-HT by blood platelets *in vitro*. Thus it seems reasonable to assume that reserpine in some way blocks the process of monoamine storage. This may well be the main site of action of the drug. The fact that reserpine behaves like a liberating agent, causing a depletion of the stores, does not contradict such a view. The two processes, storage and release, are presumably balanced in the normal cell. Thus if the storage process is blocked the result will be a depletion, the rate of which must be dependent *i. a.* on the secretory activity of the cell. In the brain the monoamines have a high turnover (UDENFRIEND and WEISSBACH 1958, CARLSSON 1960) and consequently a blocking of the storage process will rapidly cause their disappearance.

The effect of reserpine on isolated amine granules will be discussed in another paper.

The content and intracellular distribution of amines in the medulla of rabbits, to which only reserpine had been given were also studied (Table I) Very small amounts of NA and DA were present 25 hours after the injection, and stored amines appeared during the following days much more slowly than after an insulin depletion (*see* BERTLER, HILLARP *and* ROSENGREN *1960 a*) The slow recovery however may not entirely be a result of an inhibition of the storage process since this inhib tion seems to be rather weak 48 hours after the administration of reserpine as judged by the experiments with dopa injection.

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References

BERTLER, Å. A. CARLSSON and E. ROSENGREN, A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* 1958. *44* 273—292.

BERTLER, Å., N.-Å. HILLARP and E. ROSENGREN, Some observations on the synthesis and storage of catecholamines in the adrenaline cells of the suprarenal medulla. *Acta physiol. scand.* 1960 a. *50* 124—131

BERTLER, Å., N.-Å. HILLARP and E. ROSENGREN Storage of new-formed catecholamines in the adrenal medulla. *Experientia* (Basel) 1960 b. *16.* 419.

BERTLER, Å., A. M. ROSENGREN and E. ROSENGREN, I vivo uptake of dopamine and 5-hydroxytryptamine by adrenal medullary granules. *Experientia* 1960 (Basel) *16.* 418.

BRODIE, B. B., E. G. TOMICH, R. KUNTZMAN and P A. SHORE, On the mechanism of action of reserpine effect of reserpine on capacity of tissues to bind serotonin. *J Pharmacol.* 1957 *119* 461—467

CARLSSON, A., Data presented at the Ciba Found. *Symp. on Adrenergic Mechanisms.* London 1960

CARLSSON, A. E. ROSENGREN, Å. BERTLER and J NILSSON Effect of reserpine on the metabolism of catechol amines. I Psychotropic Drugs. Ed. S Garattini and V Ghetti, Amsterdam Elsevier Publ. Comp. 1957 363—372

CARLSSON A. and B. WALDECK, A fluorimetric method for the determination of dopamine (3-hydroxytyramine) *Acta physiol. scand.* 1958. *44* 293—298.

HUGHES, F B. P A. SHORE and B. B. BRODIE, Serotonin storage mechanism and i inter action with reserpine *Experientia* (Basel) 1958. *14* 178.

KIRSHNER, N Biosynthesis of adrenaline and noradrenaline. *Pharm. Rev.* 1959. *11* 350—357

KRONEBERG, G and H J SCHÜMANN, Die Wirkung des Reserpins auf den Hormongehalt des Nebennierenmarks. *Arch. exp. Path. Pharmak.* 1957 *231* 349—360.

SHORE, P A., A. PLETSCHER, E. G TOMICH, A. CARLSSON, R. KUNTZMAN and B. B. BRODIE, Role of brain serotonin in reserpine action. Ann. N Y *Acad. Sci.* 1957 *66.* 609—617

SHORE, P A., S. L. SILVER and B. B. BRODIE, Interaction of reserpine serotonin, and lysergic acid diethylamide in brain. *Science* 1955 *122* 284—285

UDENFRIEND, S. and H. WEISSBACH, Turnover of 5-hydroxytryptamine (serotonin) in tissues. *Proc. Soc. exp. Biol.* (N Y) 1958. *97* 748—751

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Phenylalanine and Tyrosine in the Adrenal Medulla

By

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Abstract

HALL, G N.-Å. HILLARP and G. THIEME. *Phenylalanine and tyrosine in the adrenal medulla.* Acta physiol. scand. 1961. 52 49—52. — The claim (FELLMAN 1958, FELLMAN and DEVLIN 1958) that large amounts of free phenylalanine are present in the adrenal medulla has been tested using cation exchange and paper chromatographic methods. The concentration of this amino acid was found to be very low (< 20 to 40 μg/g wet wt) Tyrosine — but no m-tyrosine, phenyl-serine or 3,4-dihydroxyphenylalanine (< 20 μg/g) — was detected in small amounts (10 to 20 μg/g) Thus the catecholamine producing cells in the gland do not accumulate free phenylalanine or tyrosine to level appreciably higher than that in the blood plasma.

FELLMAN and DEVLIN (1958) and FELLMAN (1958) have reported the presence of large amounts of free phenylalanine in the adrenal medulla. This finding — if correct — would suggest that the catecholamine synthesis in the gland starts from phenylalanine and not from tyrosine as generally believed.

By use of more suitable chromatographic methods than those applied by FELLMAN it is shown in this paper that the concentration of both phenylalanine and tyrosine in the adrenal medulla does not materially exceed that in the blood plasma.

Material and Methods

Extraction procedure Pooled adrenal medullas of cow were thoroughly extracted with perchloric acid (PCA final concentration 0 4 N) After centrifugation the extract was neutralized at 0° to about pH 3—4 with potassium carbonate. In some experiments extraction was performed with 0.1 N HCl according to the procedure of UDENFRIEND and COOPER (1953)

Ion exchange chromatography Two different procedures were used.

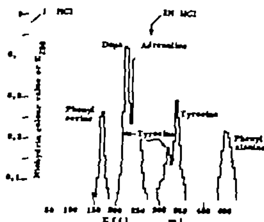


Fig. 1 Cation exchange chromatography (Dowex 50 W – X 8, 100 × 0.6 cm, H+) of phenylserine, m-tyrosine, tyrosine, phenylalanine (0.5 mg of each), dopa and adrenaline (1 mg of each).

Method I To the extract from 1 g (wet wt) of medulla, acetic acid was added to a final concentration of 1 N. The solution was transferred to a cation exchange column (Dowex 50W—X8, 200—400 mesh, 30 × 0.6 cm, H) with a small portion of N acetic acid. The amino acids were eluted (4.6 ml fractions) with 1 M acetic acid — sodium acetate buffer of pH 4.0. Ninhydrin positive substances — determined according to the method of MOORE and STEIN (1954) — were eluted in three sharp peaks. Phenylalanine and tyrosine (added to the extract) appeared in the last and biggest peak when 100 to 110 ml of the buffer had passed through the column.

Method II The extract was acidified with HCl (final concentration 1 N) and applied to a longer column (100 × 0.6 cm). The elution was performed first with 1 N HCl until the catecholamines (determined by ultraviolet absorption measurements) were completely eluted and then with 2 N HCl. Practically the whole amount of the ninhydrin positive components were recovered in various fractions of the N acid. Meta-tyrosine, tyrosine and phenylalanine (100 to 500 μg of each added to the extract) appeared in fairly sharp peaks and free from substances interfering in the paper chromatographies when about 20, 50 and 150 ml of the second eluant had passed through the column. When added to the extract, phenylserine was eluted with the N acid in sharp peak before 3 4-dihydroxyphenylalanine which emerged — together with noradrenaline — immediately before the drenaline peak. — A chromatogram of synthetic mixture of amino acids is shown in Fig. 1.

In two experiments an extract from 5 g of medulla was chromatographed according to method I on larger column (40 × 1.3 cm). All the fractions with ninhydrin positive substances of the third peak were pooled and evaporated to dryness *in vacuo* at + 40° after acidification with HCl. The residue was thoroughly extracted with 95 % ethanol containing 1 % HCl. After evaporation of most of the ethanol with N_2, the material was dissolved in N HCl and re-chromatographed according to method II after addition of 500 μg of adrenaline, used as a guide for the development of the chromatogram.

When HCl was used as eluant, aliquots of the fractions were evaporated to dryness *in vacuo* over sodium hydroxide before the ninhydrin determinations were made.

Paper chromatography The fractions containing the various peaks of ninhydrin positive substances were evaporated to dryness *in vacuo* t + 40° by use of a rotating evaporator When the acetate buffer was used as eluant the fractions were first acidified with an appropriate amount of HCl. The residue was taken up in small olume of

acid ethanol or 0.1 N HCl and applied to Whatman no. 1 paper (washed with HCl and sodium ethylenediaminetetraacetate). The material was subjected to ascending chromatography in two systems: n-butanol — glacial acetic acid — water 4 : 1 : 5 and isopropanol — aqeous ammonia (25 % NH_3) — water 80 : 2 : 18. The spots were located by spraying with a 0.2 % solution of ninhydrin in ethanol or — for location of phenolic compounds — with diazotized p-nitroaniline according to STUDNITZ and HANSON (1959). Pure amino acids (2—10 μg) were used as references.

Determination of tyrosine. After ion exchange chromatography tyrosine was determined colorimetrically in the eluted fractions using 1-nitroso-2-naphtol according to UDENFRIEND and COOPER (1952).

Recovery experiments. Only small losses, or none at all, were found on ion exchange chromatography of pure amino acids as shown by direct determinations with ninhydrin. In four experiments, tyrosine (100 μg) and phenylalanine (100—500 μg) were added to 1 g of gland tissue which was then extracted with PCA. Ion exchange (method II) and paper chromatography were performed as described above. The recovery was at least 70 %.

Results and Discussion

FELLMAN and DEVLIN (1958) found very large amounts of free phenylalanine in the beef adrenal medulla (900 to 1 000 μg/g wet wt). The chemical determination used, however, is unspecific and the present authors have found the identification of the amino acid by direct paper chromatography of an unfractionated tissue extract to be unreliable. Two cation exchange procedures were therefore devised in order to remove interfering substances. In one of them (method II) the catecholamines and essentially the whole amount of ninhydrin positive compounds and other interfering components could be removed before tyrosine and phenylalanine — added to the extract — were eluted in two well separated and fairly sharp peaks (see Fig. 1). In spite of the fact that phenylalanine added to the gland tissue before the extraction took place was easily detected and recovered in good yield, no phenylalanine was found in the adrenal medulla. Since the methods used admitted of detection of 20 to 40 μg in an extract from 1 g of medulla, the concentration of this amino acid in the gland must be very low. In fact, it is probably as low as that in other tissues and blood plasma (see STEIN and MOORE 1954, TALLAN, MOORE and STEIN 1954).

Tyrosine — but no m-tyrosine, phenylserine or 3,4-dihydroxyphenylalanine (< 20 μg/g) — was detected in small amounts. Its concentration in the gland was estimated to be in the order of 10 to 20 μg/g, i.e. about the same as that in other tissues and blood plasma.

Thus the cells in the adrenal medulla do not accumulate free phenylalanine or tyrosine to any appreciable extent. This may mean that the amino acid used as starting material for the catecholamine production is drawn directly from the blood when it is needed. It has been shown (ROSENFELD, LEEPER and UDENFRIEND 1958) that the cells can take up tyrosine from the blood and utilize it in the amine synthesis. There is thus at present no reason to believe

that this synthesis starts from phenylalanine. The fact that slices of adrenal medulla are able to convert phenylalanine to tyrosine does not contradict this view since this conversion was found to be due to a non-enzymatic and presumably non-specific reaction (FELLMAN and DEVLIN 1958)

This work has been supported by grants from the Swedish Medical Research Council, the Air Force Office of Scientific Research and Development Command, United States Air Force, and by a grant (B-2854) from the United States Public Health Service.

References

FELLMAN, J H Epinephrine metabolites and pigmentation in the central nervous system in a case of phenylpyruvic oligophrenia. *J Neurol. Neurosurg Psychiat.* 1958. *21* 58—62.

FELLMAN, J H. and M. K. DEVLIN, Concentration and hydroxylation of free phenylalanine in adrenal glands. *Biochim. biophys. Acta* 1958. *28* 328—332

MOORE, S. and W H. STEIN, A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J biol. Chem.* 1954 *211* 907—913.

ROSENFELD, G L. C. LEEPER and S. UDENFRIEND Biosynthesis of norepinephrine and epinephrine by the isolated perfused calf adrenal. *Arch. Biochem. Biophys.* 1958. *74* 252—265.

STEIN, W H. and S. MOORE, The free amino acids of human blood plasma. *J biol. Chem.* 1954. *211* 915—926.

STUDNITZ, W v and A. HANSON Determination of 3-methoxy-4-hydroxymandelic acid in urine by high-voltage paper electrophoresis. *Scand. J clin. Lab. Invest.* 1959 *11* 101—105.

TALLAN, H. H., S. MOORE and W H. STEIN Studies on the free amino acids and related compounds in the tissues of the cat. *J biol. Chem.* 1954 *211* 927—939

UDENFRIEND, S. and J R. COOPER, The chemical estimation of tyrosine and tyramine. *J biol. Chem* 1952 *196*. 227—233

UDENFRIEND S. and J R. COOPER, Assay of L-phenylalanine as phenylethylamine after enzymatic decarboxylation. *J biol. Chem.* 1953. *203*. 953—960.

Acta physiol. scand. 1961. 52. 53—61

From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

The Functional State of Vasomotor Nerves to Skeletal Muscle Vessels in Reserpinized Cats

By

SUNE ROSELL and ANDERS ROSÉN

Recieved 20 January 1961

Abstract

ROSELL, S. and A. ROSÉN. *The functional state of vasomotor nerves to skeletal muscle vessels in reserpinized cats.* Acta physiol. scand. 1961. 52. 53—61. — The functional state of vasomotor nerves to skeletal muscle vessels was studied in anaesthetized cats treated with reserpine. The effects of the vasoconstrictor nerves could be completely blocked by reserpine while the responses of the vasodilator nerves seemed to be unaffected. The results are consonant with the view that there are two types of vasomotor nerves to the skeletal muscle vessels, namely adrenergic vasoconstrictor nerves and cholinergic vasodilator nerves. The cardiovascular changes evoked by reserpine are discussed.

According to numerous investigators, there are specific vasodilator fibers in the sympathetic nerves to the skeletal muscle vessels of the cat. In view of the postganglionic nerve terminals, BÜLBRING and BURN (1935, 1936) and ROSENBLUETH and CANNON (1935) claim that both adrenergic and cholinergic vasodilator nerves exist in the cat, while FOLKOW and UVNÄS (1950) argue that the skeletal muscle vessels have only cholinergic vasodilator nerves. On the other hand, P. CANNON, RAULE and SCHAEFFER (1954) still question the existence of vasodilator nerves. They adhere to the view that vasodilatation is due to inhibition of vasoconstrictor nerve activity.

Reserpine has been found to deplete various organs of dopamine, adrenaline, noradrenaline and 5-hydroxytryptamine (CARLSSON *et al.* 1958, BERTLER, CARLSSON and ROSENGREN 1956, HOLZBAUER and VOGT 1956, SHORE, SILVER

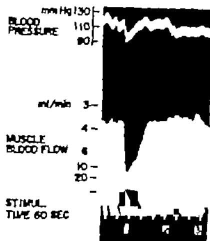


Fig. 1. Cat 3.5 kg. Chloralose Urethane. Effect on muscle blood flow of stimulation of the sympathetic chain after reserpine treatment (0.05 mg/kg/day for 3 days).
1. Stimulation, 2.5 V, 5 imp/sec.
2. Atropine, 0.5 mg/kg I. V.
3. Stimulation, 2.5 V, 5 imp/sec.
Note vasodilatation blocked by atropine.

and BRODIE 1955) BERTLER *et al.* (1956) and MUSCHOLL and VOGT (1958) have demonstrated that following reserpine treatment the adrenergic neurones lose their transmitter substance. This action of reserpine has been used in our study designed to reinvestigate whether specific vasodilator nerves exist and if so what type of postganglionic fibers they possess. Our experiments also yielded information on certain cardiovascular actions of reserpine, which will be discussed in the following.

Methods

Reserpine Treatment

From one to six days prior to the experiments, reserpine (Serpedin® Pharmacia) was administered subcutaneously in daily doses of from 0.025 to 0.5 mg/kg. In the animals which received only a single injection, 2—5 mg/kg was given s. c. 20—24 hours before the experiment.

Preparation

Cats weighing between 2.0 and 4.5 kg were anesthetized with urethane i.v. (0.4—1 g/kg b. w.). In animals used for studying vasomotor reflexes, chloralose (50 mg/kg) supplemented by 20 per cent urethane (usually 0.4—0.8 g) was administered. The trachea was cannulated. Arterial pressure was recorded by a mercury manometer or a pressure transducer (Statham P 23 AA) connected to the carotid artery. Heart rate was determined with an interval recorder via impulses from the blood pressure channel of a Grass polygraph (GOLDSCHMIDT and LINDGREN 1961). Rectal temperature was maintained at 36—37° C by radiant heat from a heating lamp. Blood flow to the musculature was recorded in the femoral artery of a skinned limb, with the aid of a silicone-filled drop counter operating an ordinate recorder (LINDGREN 1958). To maintain warmth and moistness, the skin was replaced around the muscle. A ligature around the ankle isolated the paw from the circulation. In some experiments a cross-circulation technique was used. A femoral artery in the donor animal was connected via a drop counter to the

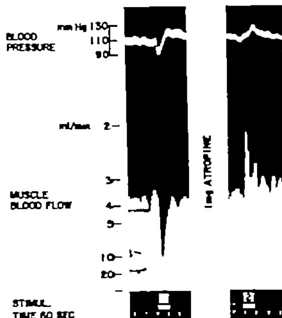


Fig. 2. Cat 2.5 kg, Chloralose-Urethane.
Effect on muscle blood flow of stimulation of the sympathetic chain after reserpine treatment (0.05 mg/kg/day for 4 days).
1 Stimulation, 2.0 V 20 imp/sec.
2. Stimulation, 2.0 V 20 imp/sec.
Note vasodilatation, after atropine, vasocontriction.

corresponding femoral artery in skinned hind limb of the recipient animal, and the blood flow to the muscles thus recorded. The cross-circulated leg was drained by a plastic tube from the femoral vein of the recipient to the corresponding vein of the donor animal. To prevent clotting, heparin (25 mg/kg) was given intravenously. All values were recorded on a Grass polygraph or on a kymograph.

The sympathetic chain, isolated and cut via the anterior approach, was stimulated in the distal part with bipolar silver electrode at the level of L_4—L_5. The carotid sinus nerve or the central part of transected vagus nerve was isolated and stimulated with bipolar silver electrode. For hypothalamic stimulation of the sympathetic vasodilator pathway unipolar stainless steel electrode, oriented by means of the Horsley Clarke technique, was used. Electrical stimulation was produced by a square-wave generator of 1,000 ohms output resistance. The duration of impulses, the voltage and the frequency were independently variable. Dextran (Macrodex® Pharmacia) was administered intravenously as required, to compensate for blood loss. Artificial respiration was given during reflex activations.

Results

The results are based on data from 51 experiments. In 34 of these the sympathetic chain was stimulated. Vasomotor reflexes were produced in 14 cats, and in 3 the sympathetic vasodilator pathway in the hypothalamus was activated.

1 *Sympathetic Chain Stimulation*

In cats, stimulation of the distal portion of the cut sympathetic chain at the level of L_4 — L_5 usually causes contriction of the skeletal muscle blood vessels



Fig 3 Cat 3 0 kg Urethane.
Effect on muscle blood flow of stimulation of the hypothalamic vasodilator pathway in reserpinized cat (5 mg/kg)
1 Stimulation, 2.0 V 70 imp/sec.
2 Stimulation, 2.0 V 70 imp/sec.
3. Atropine, 0.4 mg/kg I V
4 Stimulation, 2.0 V 70 imp/sec.
5. Bilateral drenalectomy
6. Stimulation, 2.0 V 70 imp/sec.
7 Stimulation, 2.0 V 70 imp/sec.
Note vasodilatation partly blocked by atropine after bilateral adrenalectomy no changes in blood flow

of the hind limb In reserpine-treated cats, however the same stimulation produced vasodilatation when the reserpine dose was adequate Fig 1 illustrates an experiment in which the animal received daily doses of reserpine 0.05 mg/kg for 5 days. Sympathetic chain stimulation caused pronounced vasodilatation which was blocked by atropine — a finding which suggests the existence of cholinergic vasodilator nerves. The fact that sympathetic chain stimulation caused a blood flow increase does not preclude the possibility that the transmission mechanism at the adrenergic vasoconstrictor nerve endings was still functioning, since the effect of vasoconstrictor nerve stimulation may be concealed by the increased blood flow That this was not the case however is evident from the fact that stimulation following atropine *did not* reduce the blood flow An example of vasoconstriction with repeated stimulation after atropine, is illustrated in Fig 2 In this experiment the vasodilator effect predominated even though the effect of vasoconstrictor nerve stimulation remained the dose of reserpine (0 05 mg/kg/day for 4 days) apparently having been insufficient to abolish it completely Following high doses of reserpine (2—5 mg) the effects of vasoconstrictor nerve stimulation were always *abolished*.

2 Hypothalamic Stimulation

The sympathetic vasodilator system has relay stations between the cerebral cortex and the peripheral outflow Synapses are thought to exist in the hypothalamus and in the mesencephalon (Eliasson, Lindgren and Uvnäs 1954, Lindgren 1955) *In order to ascertain whether the hypothalamic-spinal part of the vasodilator system is functionally intact or blocked at some point in reserpine-treated animals,* the vasodilator pathway was stimulated in the anterior hypothalamus of reserpinized cats (5 mg/kg) Stimulation still evoked vaso-

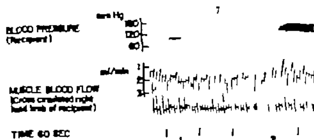


Fig 4 Cat 3.2 kg Chloralose-Urethane.
Effect on blood pressure and muscle blood flow of stimulation of the carotid sinus nerve in reserpinized cat (5 mg/kg)
1 Stimulation, 3 V 10 imp/sec.
2 Stimulation, 3 V 15 imp/sec.
Note no change in muscle blood flow; decrease in blood pressure.

dilatation which was blocked by atropine, thus indicating activation of cholinergic vasodilator nerves (Fig 3) Although stimulation following atropine still increased the blood flow the latency was now longer indicating that a humoral factor was involved. After bilateral adrenalectomy stimulation elicited no change in blood flow It seems, therefore, that even when the animal had received a large dose of reserpine (5 mg/kg) catechol amines might have been released from the adrenals by hypothalamic stimulation. This is consistent with th finding of MUSCHOLL and VOGT (1958) that even high doses of reserpine do not entirely deplete the cat s adrenal medulla of its catechol amines.

3. Reflex Activation

After having demonstrated that the vasodilator pathway was intact in reserpinized cats from the hypothalamus to the peripheral termination but that the adrenergic vasoconstrictor effects were blocked we initiated a series of experimental attempts to increase muscle blood flow reflexly In the event of such increase, vasodilatation would be attributable to activation of specific cholinergic vasodilator nerves rather than to inhibition of vasoconstrictor tone

Reflex activation was produced by afferent stimulation of one of the carotid sinus nerves or one of the vagi. In order to prevent a blood-pressure fall from influencing the blood flow in the peripheral area, a cross-circulation technique was utilized. In the experiment shown in Fig 4 the skinned right hind limb was *cross circulated. The recipient animal in this case had* received reserpine (5 mg/kg) the day before the experiment. The fact that stimulation of the carotid sinus nerve did not alter the blood flow means that the sympathetic vasodilator nerves were not activated. On the other hand, there was a blood-pressure fall in the recipient cat, indicating either bradycardia or a reduction of peripheral resistance in vascular areas other than skeletal muscle. — Studies on skin and intestinal blood flow indicate that in those areas as well, no increase of blood flow can be induced by afferent stimulation of the vagus or carotid sinus nerve after reserpine treatment (ROSELL and ROSÉN, unpublished observa-

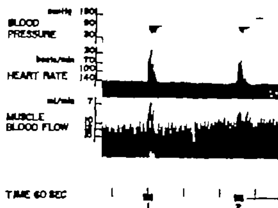


Fig 5 Cat 4.2 kg Chloralose-Urethane. Effect on heart rate and muscle blood flow of stimulation of the carotid sinus nerve in reserpinized cat (5 mg/kg)
1 Stimulation, 2.5 V 15 imp/sec.
2. Stimulation, 2.5 V 10 imp/sec.
Note reflex bradycardia.

tions) — However in reserpinized cats carotid sinus nerve stimulation induced bradycardia (Fig 5) Thus the blood-pressure fall seen in Fig 4 presumably results from a reflexly induced bradycardia.

Discussion

Sympathetic chain stimulation in reserpine-treated cats produces an increased blood flow in skeletal muscle vessels (Fig 1) in marked contrast to the vasoconstrictor response usually seen in untreated cats. The disappearance of the vasoconstrictor response is presumably due to loss of the transmitter substance from the adrenergic neurones following reserpine treatment (BERTLER *et al.* 1956 MUSCHOLL and VOGT 1958) If this loss is of sufficient magnitude adrenergic nerve impulses will be incapable of producing responses in the effector organ Thus, if cholinergic vasodilator nerves exist, their effects should predominate when the sympathetic chain is stimulated Even relatively small doses of reserpine (0 05 mg/kg/day for 4 days) are sufficient to cause predominance of the effects of the vasodilator nerves (Fig 2) When the cholinergic vasodilator nerve response has been blocked by atropine, renewed sympathetic stimulation provides information about the functional state of the adrenergic constrictor nerves (Fig 1 and 2)

It seems evident that reserpine affects the adrenergic vasomotor neurones peripherally Investigations by others authors, using different methods, lead to similar conclusions. BERTLER *et al.* (1956) showed that stimulation of the sympathetic ganglia by carbacholine failed to increase the blood pressure of atropinized cats treated with reserpine. This may indicate that the transmission mechanism at the adrenergic nerve endings was no longer intact, due possibly to the depletion, by reserpine, of the transmitter substance in the

adrenergic nerves. MUSCHOLL and VOGT (1958) have shown, moreover that peripheral adrenergic neurones lose their noradrenaline after injection of reserpine.

Fig 1 shows that sympathetic chain stimulation induces vasodilatation in cats treated with reserpine and that the vasodilatation is blocked by atropine. These facts strongly indicate the existence of specific cholinergic vasodilator nerves to the skeletal muscle vessels. This is contradictory to the assumption that in the cat most of the sympathetic vasodilator fibers to the skeletal muscles are adrenergic (BÜLBRING and BURN 1935, 1936, ROSENBLUETH and CANNON 1935) but supports the view of FOLKOW and UVNÄS (1950) that the sympathetic vasodilator nerves are cholinergic.

P CANNON *et al.* (1954) have questioned the existence of specific vasodilator nerves and have argued that the vasodilator effects during stimulation of the sympathetic system stem from inhibition of vasoconstrictor nerve activity. This explanation appears to be invalidated by our experiments. The cross-perfusion experiments in which afferent stimulation of the carotid sinus nerve or vagi was applied, show that when the dose of reserpine is sufficient there is no vasoconstrictor nerve activity which can be inhibited. It is still possible, however to induce vasodilatation of skeletal muscle vessels by sympathetic chain stimulation or by appropriate stimulation in the hypothalamus.

Thus these experiments support the view of LINDGREN and UVNÄS (1955) that skeletal muscle vasodilatation may be elicited via sympathetic nerves in two different ways namely by inhibition of the adrenergic vasoconstrictor nerve activity and by activation of cholinergic vasodilator nerves. They also lend weight to the theory that sympathetic vasodilator nerves are not involved in depressor reflexes elicited from the carotid sinus region or from the aortic arch (Fig 4)

It is interesting to note in cats treated with high doses of reserpine (5 mg/kg) that the vasodilator pathway is intact from the hypothalamus to the peripheral outflow despite the fact that the central nervous system is presumably depleted of dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine. It has been suggested that some of these biogenous substances might function as chemical mediators within the central nervous system (BRODIE and SHORE 1956) Within the peripheral nervous system reserpine blocks the transmission at the adrenergic nerve endings by releasing the transmitter substance. By analogy one might assume that the transmission would also be blocked at the synapses in the central nervous system, where any one of the substances released by reserpine might act as a chemical mediator It is doubtful therefore, whether any of the afore mentioned substances functions as a chemical mediator within the sympathetic vasodilator system.

The fact that afferent stimulation of the vagus or of a carotid sinus nerve produces no reflex vasodilatation in reserpinized cats could be explained by SCHNEIDER's (1955) hypothesis that reserpine blocks afferent stimuli, preventing

them from reaching sympathetic centers, or by BEIN's (1953 and 1955) suggestion that reserpine activates central inhibitory sympathetic structures. Both authors found that after administration of reserpine the blood-pressure response to activation of the carotid occlusion reflex was diminished. HORWITZ, KUBERT and WANG (1959) recently reported that pressor responses from medullary and hypothalamic vasomotor areas were reduced in reserpine treated cats. They postulate a general depressive effect of reserpine on the central vasomotor mechanism. ANAND, DUA and MALHOTRA (1957) and HARRISON and GOTH (1956) hold, in general, a similar view concerning the action of reserpine. The results of these experiments might well be explained by partial or total inactivation of peripheral adrenergic efferent neurones. Since the carotid occlusion reflex not only affects the vasomotor tone of peripheral vessels but also changes the heart rate, it is difficult to analyse the underlying mechanism of reserpine's circulatory effects by studying changes in blood pressure. To judge from our results, reserpine does not interfere with transmission in the synapses which relay impulses to the efferent part of the reflex arc studied, since stimulation of the carotid sinus nerve invariably induced pronounced bradycardia. That reserpine reduces the blood-pressure response during the carotid occlusion reflex, may be explained by the fact that the reflex increase in vasoconstrictor tone and in heart rate is diminished or precluded because the efferent adrenergic neurones are no longer intact.

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References

ANAND, B. K., S. DUA and C. L. MALHOTRA, Effects of reserpine on blood-pressure responses evoked from the hypothalamus. *Brit. J. Pharmacol.* 1957. *12.* 8—11.

BEIN, J. H. Zur Pharmakologie des Reserpin, eines neuen Alkaloids aus Rauwolfia serpentina Benth. *Experientia* (Basel) 1953. *9.* 107—110.

BEIN, J. H. Significance of Selected Central Mechanism for the Analysis of the Action of Reserpine. *Ann. N. Y. Acad. Sci.* 1955. *61.* 4—16.

BERTLER, Å., A. CARLSSON and E. ROSENGREN, Release by reserpine of catechol amines from rabbit hearts. *Naturwissenschaften* 1956. *43.* 521.

BRODIE, B. B. and P. A. SHORE, A concept for role of serotonin and norepinephrine as chemical mediators in the brain. *Ann. N. Y. Acad. Sci.* 1956. *66.* 631—642.

BÜLBRING, E. and J. H. BURN, The sympathetic dilator fibres in the muscle of the cat and dog. *J. Physiol.* (Lond.) 1935. *83.* 483—501.

BÜLBRING, E. and J. H. BURN, The Sherrington Phenomenon. *J. Physiol.* (Lond.) 1936. *86.* 61—76.

CARDON, P., W. RAULE and H. SCHAEFER, Zur Physiologie eines sympathischen Ganglions und zur Frage der Vasodilatatoren und des sympathischen Tonus. *Pflügers Arch. ges. Physiol.* 1954. *260.* 116—136.

CARLSSON, A., M. LINDQVIST, T. MAGNUSSON and B. WALDECK, On the presence of 3-hydroxytyramine in brain. *Science* 1958. *127.* 471.

ELIASSON, S., P LINDGREN and B. UVNÄS, The hypothalamus, a relay station of the sympathetic vasodilator tract. *Acta physiol. scand.* 1954. *31* 290—300.

FOLKOW B. and B. UVNÄS, Do adrenergic vasodilator nerves exist? *Acta physiol. scand.* 1950. *20* 329—337

GOLDSCHMIDT H. and P LINDGREN, An electronic interval recorder for measuring peripheral blood flow and heart rate *J appl. Physiol.* 1961 To be published.

HARRISON, F and A. GOTH, Effect of reserpine on the hypothalamic pressor response. *J Pharmacol.* 1956. *116* 262—267

HOLZBAUER, M. and M. VOGT Depression by reserpine of the noradrenaline concentration in the hypothalamus of the cat. *J Neurochem.* 1956. *1* 8—11

HORWITZ, B., S. KUNTZ and S. C. WANG, Mechanism of hypotensive action of reserpine. *Arch. int Pharmacodyn.* 1959 *120* 229—242.

LINDGREN, P and B. UVNÄS, Vasoconstrictor inhibition and vasodilator activation. — Two functionally separate vasodilator mechanisms operating in skeletal muscles. *Acta physiol. scand.* 1955 *33* 108—119.

LINDGREN P The mesencephalon and the vasomotor system. *Acta physiol. scand.* 1955. *35* Suppl. 121 1—189

LINDGREN, P An improved method for drop recording of arterial or venous blood flow *Acta physiol. scand.* 1958. *42* 5—11

MUSCHOLL, A. and M. VOGT The action of reserpine on the peripheral sympathetic system *J Physiol.* (Lond.) 1958. *141* 132—155.

ROSENBLUETH, A. and W B. CANNON, The chemical mediation of sympathetic vasodilator nerve impulses. *Amer J Physiol.* 1935. *112* 33—40.

SCHNEIDER, J A., Further characterization of central effects of reserpine (Serpasil) *Amer J Physiol.* 1955. *181* 64—68.

SHORE, P A., S. L. SILVER and B. B. BRODIE, Interaction of reserpine, serotonin, and lysergic acid diethylamide in brain. *Science* 1955. *122* 284—285.

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From the Physiology Department, Karolinska Institutet, Stockholm, Sweden

Occurrence and Distribution of Catecholamines in the Fish Brain

By

U. S. von Euler

Received 24 January 1961

Abstract

Euler, U. S. v. *Occurrence and distribution of catecholamines in the fish brain.* Acta physiol. scand. 1961. *52.* 62—64. — Relatively high amounts of catecholamines were found in the brains of an elasmobranch, *Squalus acanthias* and a teleost, *Gadus callarias*. Only small quantities could be demonstrated in the brain of a cyclostome, *Myxine glutinosa*. Both adrenaline and noradrenaline occurred in the brains of *Squalus* and of *Gadus* although noradrenaline was predominant. A characteristic distribution of the catecholamines in the brain of *Squalus* was observed. The highest amount of noradrenaline was found in the diencephalon, hypothalamus, and hypophysis, while the cerebellum contained only very small quantities.

Noradrenaline and small amounts of adrenaline have been demonstrated in the brain of mammals (cf. Euler 1956). Vogt showed in 1954 that certain regions of the brain like the hypothalamus contained larger amounts of amines than others. It is still uncertain whether all of the noradrenaline in the brain is present in postsynaptic neurons, *e. g.* vasomotor fibres, or whether part of it occurs in chromaffin cells or in other stores. As to the adrenaline it may be postulated that it is present in chromaffin cells, since there is no evidence for its occurrence in axons.

The present study was made in order to find out whether catecholamines also occurred in fish brain and, if this were the case, to obtain data on their distribution.

Methods

Brains were prepared from cyclostome (*Myxine glutinosa*, hagfish) an elasmobranch (*Squalus acanthias*, dogfish) and teleost (*Gadus callarias*, cod) in freshly killed specimens. The brains of *Myxine* and *Gadus* were pooled in sufficiently large quantities to allow accurate estimations of the amines. After weighing, the brains were ground in a mortar with 10 per cent trichloroacetic acid, and filtered after 1/2 hour extraction. The residue

Table I. Adrenaline and noradrenaline in fish brains (total)

	n	Adr µg/g	Noradr. µg/g	Per cent adr
Myxine glutinosa (Cyclostome)	15	< 0.02	< 0.02	–
Squalus acanthias (Elasmobranch)	8	0.11	0.37	23
Gadus callarias (Teleost)	5	0.03	0.27	10

Table II Adrenaline and noradrenaline in the brain of Squalus acanthias (dogfish) (6 animals)

Part of brain	Total weight g	Adr µg/g	Noradr µg/g	Adr %
Telencephalon	5.2	0.12	0.49	20
Optic lobes.	3.3	0.20	0.31	39
Diencephalon.	0.77	0.31	0.53	36
Hypothalamus	0.62	0.33	0.44	43
Pituitary gland	0.38	0.41	1.5	22
Cerebellum.	3.0	0.011	0.056	16
Medulla oblongata	3.6	0.13	0.25	34
Total brain (6 animals)	17.1	0.14	0.35	29
Total brain (2 animals)	4.4	0.072	0.39	16

was washed and the remaining solvent pressed out. The reaction was adjusted to pH 8.2—8.5 with 1 N Na OH and the catecholamines adsorbed on an alumina column, eluted with 0.25 N acetic acid and estimated fluorimetrically according to EULER and LISHAJKO (1959).

The brains of 6 specimens of *Squalus* were divided in 7 portions (telencephalon, optic lobes, diencephalon, hypothalamus, hypophysis, cerebellum and medulla oblongata) and extracted for catecholamines as described above.

Results

The results are given in Table I and II showing the amounts of adrenaline and noradrenaline in the whole brain of the three types of fish studied, and their distribution in various parts of the brain of the dogfish.

As seen in Table I the amount of catecholamines is very small in the *Myxine* brain while it may be considered as relatively high in *Squalus* and *Gadus* being of the same order of magnitude as in mammalian brain.

The relative amounts of noradrenaline and adrenaline also show characteristic differences in the different kinds of fish, adrenaline being relatively higher in *Squalus* than in the teleost studied.

Table II gives the absolute and relative amounts of adrenaline and noradrenaline in the different parts of the brain of *Squalus*. As seen in the table the catecholamine amounts are highest in the diencephalon, in hypothalamus, and in the hypophysis while they are particularly low in the cerebellum.

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From the Physiology Department, Karolinska Institutet, Stockholm, Sweden

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Abstract

EULER, U. S. v., *Occurrence and distribution of catecholamines in the fish brain.* Acta physiol. scand. 1961. 52. 62—64. — Relatively high amounts of catecholamines were found in the brains of an elasmobranch, *Squalus acanthias* and a teleost, *Gadus callarias*. Only small quantities could be demonstrated in the brain of a cyclostome, *Myxine glutinosa*. Both adrenaline and noradrenaline occurred in the brains of *Squalus* and of *Gadus* although noradrenaline was predominant. A characteristic distribution of the catecholamines in the brain of *Squalus* was observed. The highest amount of noradrenaline was found in the diencephalon, hypothalamus, and hypophysis, while the cerebellum contained only very small quantities.

Noradrenaline and small amounts of adrenaline have been demonstrated in the brain of mammals (*cf* EULER 1956). VOGT showed in 1954 that certain regions of the brain like the hypothalamus contained larger amounts of amines than others. It is still uncertain whether all of the noradrenaline in the brain is present in postsynaptic neurons, *e g* vasomotor fibres, or whether part of it occurs in chromaffin cells or in other stores. As to the adrenaline it may be postulated that it is present in chromaffin cells, since there is no evidence for its occurrence in axons.

The present study was made in order to find out whether catecholamines also occurred in fish brain and, if this were the case, to obtain data on their distribution.

Methods

Brains were prepared from a cyclostome (*Myxine glutinosa*, hagfish) an elasmobranch (*Squalus acanthias* dogfish) and a teleost (*Gadus callarias* cod) in freshly killed specimens. The brains of *Myxine* and *Gadus* were pooled in sufficiently large quantities to allow accurate estimations of the amines. After weighing, the brains were ground in a mortar with 10 per cent trichloroacetic acid, and filtered after 1/2 hour extraction. The residue

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Studies on the Transfer of Phosphate from Mother to Foetus in the Rabbit

By

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Abstract

FUCHS, A. R. and F. FUCHS. *Studies on the transfer of phosphate from mother to foetus in the rabbit.* Acta physiol. scand. 1961 52 65—74. — The transfer of phosphate from mother to foetus was studied in rabbits with radioactive P. As in guinea pigs the inorganic phosphate concentration is higher in the foetal than in the maternal plasma, and the transfer takes place against the gradient. The transfer rates calculated from experiments of 30 min duration did only exceed or approach the rate of foetal P retention when the foetal and placental uptake rates were combined. In an experiment of 120 min duration the foetal uptake alone equalled the foetal retention. The combined foetal and placental uptake rates in experiments of varying duration were relatively constant.

In some of the rabbits gestation was artificially prolonged by daily injections of progesterone. The foetal P concentration increases up to but not beyond term. The placental concentration is fairly constant in the second half of gestation and after term, but goes up when intrauterine foetal death occurs, indicating that the placenta stays alive for some time and continues to take up phosphate. The active transport mechanism in the placenta must be localized to the chorionic cells, probably t their boundary toward the intervillous space, building up in the placenta phosphate pool, from which the foetus is supplied.

In previous work on the placental transfer of phosphate in the guinea pig (FUCHS and FUCHS 1957 a, b, c, FUCHS 1957) we found that inorganic phosphate is transferred from the maternal to the foetal plasma across the placental barrier against a concentration gradient, and that the placental tissue contains considerable amounts of inorganic phosphate, forming a pool from which the foetus is supplied. The rates of phosphorus uptake in the foetus and in the placenta from the maternal plasma phosphate were measured with the aid of

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radioactive phosphorus, and it was found that these two rates had to be combined to cover the rate of foetal deposition of phosphorus, as determined chemically by ashing foetuses of different gestation ages. It was further found that there is some return of phosphate from the foetus to the mother and that the safety factor *i.e.* the ratio between the rate of foetal phosphorus uptake from maternal plasma phosphate and the rate of foetal phosphorus deposition, became very low less than 1.5 at the end of gestation.

It was decided to carry out a series of experiments in the rabbit with the same technique in order to find out whether the placental transfer of phosphate follows the same pattern in this animal. In addition, it was found of interest to study the phosphate transfer in artificially prolonged gestation to see whether a placental insufficiency occurred after term. The results of the study of prolonged pregnancies have been discussed elsewhere (FUCHS and FUCHS 1960) and will only be briefly mentioned here.

Methods

Virginal Danish White Land Rabbits of uniform breed and of approximately the same age and weight were mated under supervision. From previous studies (FUCHS and FUCHS 1958) the average length of gestation was known to be around 31 days.

The rabbits to be studied after term were given 5 mg progesterone in oil intramuscularly from a gestation age of 29 days plus 6 to 12 hours and daily until the operation one to 6 days later. The main group of rabbits were not given progesterone. They were operated upon between the 18th day of gestation and term.

The transfer of phosphate to the foetuses was studied with the aid of radioactive phosphorus (P^{32}) which was injected in the form of inorganic phosphate with high specific activity into an ear vein of the mother animal. After a certain period of time, generally 30 min, abdominal hysterotomy was performed and the foetuses and the placentas including the subplacentas were removed. Care was taken to avoid blood loss the umbilical vessels or the placenta. Usually four foetuses and four placentas were ashed individually for total phosphorus and radiophosphate determinations. When the litter contained more than four foetuses it was attempted to collect blood from the umbilical vein of the remaining foetuses. During the experiment a number of blood samples were withdrawn from the carotid artery of the mother to determine the rate of disappearance of radiophosphate from the plasma. The concentration of inorganic phosphate was determined in the maternal plasma in each experiment and in the foetal plasma when available. The determination of plasma phosphate, total foetal and placental phosphorus, and radioactive phosphorus were carried out as previously described (FUCHS and FUCHS 1957 a, FUCHS 1957).

The amount of inorganic phosphate transferred to the product of conception was calculated as previously described from the amount of radiophosphate found in the foetus and placenta at the end of the experiment and the average specific activity of maternal plasma phosphate during the period.

Results

The activities of the blood samples from different experiments were plotted in the same diagram by expressing the results as relative activities, 100 per cent denoting the activity of one ml of plasma immediately after injection. This

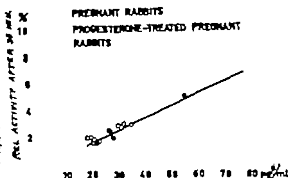


Fig. 1 Relation between the relative activity in plasma 35 min after intravenous injection of radioactive phosphate and the concentration of inorganic phosphorus in the plasma.

activity is calculated from the injected amount of radioactivity and the plasma volume, which according to COURTICE (1943) is 50 ml per kg body weight. When the disappearance curve from rabbits in the last half of pregnancy obtained from the present experiments, is compared with values from non-pregnant rabbits, calculated from HEVESY and HAHN (1940) the rate of disappearance ppears to be faster in the pregnant animals.

The concentration of inorganic phosphate in the maternal plasma varied between 18 and 36 μg P/ml in normal pregnancies. The disappearance rate was found to be inversely related to the concentration of inorganic phosphate. This is illustrated by Fig 1 in which the relative activities 35 min after intravenous injection are plotted against the phosphate concentration. The lower the concentration the faster the disappearance, indicating a fairly constant turnover of the plasma phosphate. No relation could be found between the maternal plasma phosphate concentration and gestation age before term, but in the prolonged pregnancies the concentration increases significantly after the 32nd day The foetal plasma concentration was determined in two cases, both on day 29 to 80 and 95 μg P/ml respectively

If pregnancy is prolonged, the foetal mortality increases for every day past term (CSAPO 1955 FUCHS and FUCHS 1958) In the present material 2 foetuses out of 10 were found dead in a litter removed 33 days after mating At 34 days + 2 hours a whole litter of 6 were alive, whereas litters removed at 34 days + 18 hours and at 35 days, with 3 5, and 9 foetuses, were all found dead in the uterus.

The average total phosphorus content of the foetuses and the average foetal weight with dvancing gestation is shown in Fig 2. From day 27 there are great variations, both within a litter and between the litters, and the size of the litter has a marked influence upon the average weight and phosphorus content of the foetuses. In prolonged pregnancies the foetuses continue to grow at approximately the same rate as at term, and they also increase their total phosphorus content.

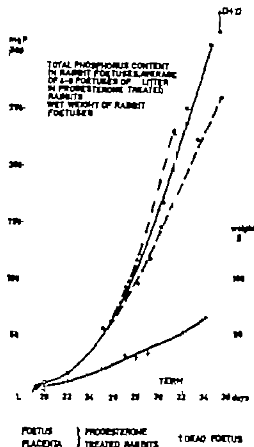


Fig. 2. Total phosphorus content and weight of rabbit foetuses at various stages of gestation. The broken lines indicate the variation of the litter averages during the last week of gestation.

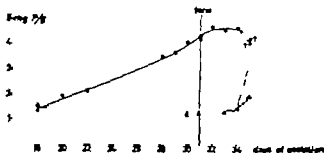
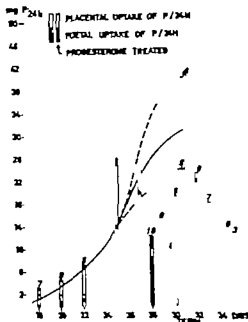


Fig. 3. Foetal and placental phosphorus content per g wet weight in the last half of gestation and in prolonged pregnancies.

If the mean foetal phosphorus concentration is considered (Fig. 3) it shows a steady increase until term, but after term the foetus does not get enough phosphorus to increase the concentration further. This finding is significant, because it was found that the foetal phosphorus concentration is very constant within a litter regardless of its size, and depends only upon the gestation age.

Fig 4. Placental uptake (open columns) and foetal uptake (filled columns) of inorganic phosphate per 24 h as determined from radiophosphate experiments of 30 min duration, compared with the daily phosphorus retention in the foetus as calculated from Fig 2. The solid line indicates the mean retention, the broken lines the limits of variation of litter averages. The numbers at the columns indicate litter size.



The weight of the blood filled placentas was about 4 g at day 18 and increased until day 25 after which it remained fairly constant around 7 g The placental phosphorus concentration remains almost constant during the whole second half of gestation and the first days after term. When the foetuses die, there is a sudden accumulation of phosphorus in the placentas, and consequently the concentration goes up (Fig 3)

From the radiophosphate data the daily transfer of phosphate from mother to foetus can be calculated (Fig 4) The lower part of each column shows the uptake per foetus which increases from 0.2 mg/24 h on day 18 to 30 mg/24 h at term. After term there is no further increase, the daily uptake seems to be fairly constant until the foetuses die. From day 27 the litter size has a marked influence upon the foetal phosphate uptake. The comparatively highest uptake per foetus is usually found in the smallest litters.

The rate of placental uptake of phosphate, indicated by the upper part of the columns in Fig 4 is considerable already 18 days after mating namely 3.2 mg/24 h as compared with the foetal uptake of 0.2 mg/24 h. The increase with advancing gestation is less, however and occurs only until day 25 after which t remains fairly constant with an average of 7 4 mg/24 h. In the three cases with dead litters around day 35 foetal death appeared to have occurred a considerable period of time before in one case, and the partly separated placentas were not examined. In the two other cases the placentas appeared to be intact and the rat of uptake was as high as about 13 mg/24 h.

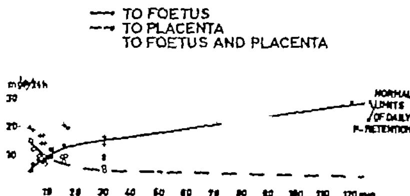


Fig. 5. Foetal and placental uptake of phosphate per 24 h, calculated from radiophosphate experiments of duration varying from 4 to 120 min, but all performed on day 29. The horizontal lines indicate the limits of foetal phosphorus retention per 24 h on this particular day of gestation. From the experiments of short duration the individual foetal and placental values are plotted, from those of 30—120 min duration only the averages.

The curves on Fig. 4 indicate the average daily phosphorus retention per foetus, as calculated from the data shown on Fig. 2. The broken lines show the variation limits of the litter averages and the solid line the calculated mean phosphorus retention. In the placentas no deposition of phosphorus takes place after day 25 except in case of intrauterine foetal death.

It is seen that the daily foetal uptake of phosphate as measured with radiophosphate in experiments of 30 min duration is definitely below the daily foetal phosphorus retention as measured by chemical analysis. This discrepancy is undoubtedly due to the presence of a placental phosphorus pool with which the phosphate becomes mixed during its passage from the maternal blood to the foetus. It is apparent that a better agreement with the foetal rate of retention obtained when the foetal and placental rates of uptake are combined.

Further experiments were therefore carried out to elucidate the effect of the duration of the experiment on the calculated foetal and placental uptake of radiophosphate. Six rabbits were injected with radioactive phosphate at the same stage of gestation, 29 days after mating but the foetuses and placentas were removed after different periods of time, varying from 4 to 120 min. The results, including the 30 min experiment on day 29 from the first series, are shown on Fig. 5. From the experiments of short duration the individual foetal and placental values are plotted, from those of 30 min and upwards only the averages. The calculated foetal rate of uptake is low in experiments of short duration, but increases to reach the rate of daily phosphorus retention in experiments of 2 hours duration. The placental rate of uptake, on the other hand, decreases with increasing duration of the experiment. The sum of the foetal and placental uptake rates was found to be fairly constant irrespective of the duration of the experiment, and of the same order of magnitude as the daily phosphorus retention.

The rapidly decreasing specific activity of the maternal plasma phosphate during the first minutes after intravenous injection of radiophosphate constitutes a considerable source of error in the calculations of the average specific activity during the experiment. This is probably the explanation of the failure of the combined foetal and placental uptake rates to reach the daily phosphorus retention in the very short experiments in Fig 5 In some cases the failure may be attributed to a particularly large litter size In such cases the rate of uptake per (foetus + placenta) might be expected to be below that in a case of average litter size. The two rabbits in Fig 5 delivered after 30 min had 7 and 9 foetuses, respectively as compared with 3—6 foetuses in the other litters in this series.

Discussion

The placental transfer of inorganic phosphate in the rabbit follows the same pattern as previously shown in the guinea pig In both species the foetal rate of uptake of phosphate as measured with radiophosphate in experiments of 30 min duration is too low to account for the phosphorus retention actually taking place. In the guinea pig the addition of the placental uptake rate brought the total uptake of the product of conception considerably above the rate of phosphorus retention, the safety factor being about 8 in the middle of gestation and 1.5 at the end. In the rabbit the calculated foetal rate of uptake increases with increasing duration of the experiment and becomes equal to the phosphorus retention rate in 2 hours experiments. The combined foetal and placental uptake rates are independent of the duration of the experiment (at least up to 2 hours) This justifies the use of the combined uptake rates for the calculation of the transferred amounts of phosphate.

These results together with the fact that the placenta continues to take up phosphate from the maternal plasma and increases its phosphorus concentration after cessation of the foetal circulation indicate the presence of a phosphorus pool in the placenta of the rabbit. The same is the case in the guinea pig, in which we demonstrated a high placental concentration of inorganic phosphate (from 0.25 to 0.55 mg/g wet weight) Together with some labile organic phosphates it obtained a specific activity between that of the maternal and that of the foetal plasma phosphate. The active mechanism required for the up-hill transport of inorganic phosphate from the lower maternal to the higher foetal concentration of phosphate in the plasma must therefore be located to the chorionic cells, probably at their boundary toward the intervillous space. Bothwell *et al.* (1958) have found that also the placental uptake of iron in the rabbit is an active process independent of the foetus.

Our findings indicate that in the rabbit there is no safety margin for the transfer of phosphate in the second half of gestation. During the last f ur days before term the combined uptake rates even failed to reach the average phosphorus retention in most cases.

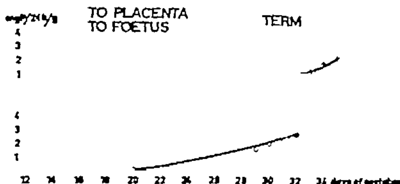


Fig. 6. Daily transfer of phosphate to foetus and placenta per g wet weight of the placenta at various stages of gestation, as calculated from radiophosphate experiments of 30 min duration.

If considerable amounts of radiophosphate return to the mother during the experiment, the calculated transfer rates would be too low but the results shown in Fig. 5 where the combined uptake is the same in short and long experiments, speak against this possibility. Probably the explanation of the low rates of uptake observed at the end of gestation is partly that the four cases in question had large litters, namely 10, 9, 8, and 6 foetuses, respectively and partly that certain errors are inherent in the determination of the average specific activity of the maternal plasma phosphate during the experimental period. The precipitous fall in the specific activity within the first minutes after the injection obviously gives rise to a considerable inaccuracy in the calculation of the average in experiments of very short duration. Experiments of 30 min duration should offer opportunities for calculation of a more reliable average, and a priori there is no reason to expect the error to vary with the stage of gestation. By our way of calculation of the average specific activity a variation of this kind may however be expected, since all calculations were based on the same standard disappearance curve, constructed from data of experiments performed at different stages of gestation. Now with the increasing foetal uptake of phosphorus towards term the rate of disappearance of radiophosphate from the maternal plasma is probably greater at the end of gestation than that indicated by the standard curve. It is therefore likely that, by using the standard curve, the transfer rates have been somewhat underestimated in this part of gestation. We feel that this together with the occurrence of large litters, may well account for the fact that (foetal + placental) uptake rates below the retention rates were observed in the experiments covering this period.

Organic phosphorus compounds or other pathways may participate in the supply of phosphorus to the foetus, but granting the correctness of the above explanation of the low uptake rates towards term, it may be concluded that inorganic phosphate is transferred from the maternal plasma in sufficient

amounts to account for the foetal phosphorus deposition in most cases. At the end of gestation the foetuses may not always get enough and consequently develop at less than the optimal rate. This supposition is supported by the observed variations in foetal size which are much greater than the variations in the guinea pig

If the rates of phosphate transfer to the foetuses and placentas are calculated per unit weight placenta, the results shown in Fig 6 are obtained. Per gram placenta the foetal uptake increases from 0.05 mg/24 h at day 18 to about 2.5 mg/24 h at term. During this period the placental barrier becomes thinner and its surface increases. The volumes of maternal and foetal blood in the placenta undoubtedly increase, as was demonstrated in the guinea pig (FUCHS 1953) and probably also the blood flow All these morphological and physiological factors facilitate the increase of the transfer That the demands on the placenta are greater in the rabbit than in the guinea pig is illustrated by the fact that the ratio foetal weight to placental weight increases by a factor of 20 in the last 13 days of the rabbit gestation and only by a factor of 5 in the corresponding period of the much longer gestation of the guinea pig, the last 26 days.

The rabbit and the guinea pig both have hæmochorial placentas with three layers of tissue separating the foetal blood from the maternal blood stream, namely chorion, connective tissue in the villi, and foetal endothelium. The human placenta belongs to the same morphological group, and another similarity of the human being is the fact that the concentration of inorganic phosphate is higher in the foetal plasma than in the maternal plasma. It would seem likely that the placental transfer of phosphate follows the same pattern in the human as demonstrated in the rabbit and the guinea pig

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References

BOTHWELL, T H., W F PRIBILLA, W MEBUST and C. A. FINCH, Iron metabolism in the pregnant rabbit. *Amer J Physiol.* 1958. *193.* 615—622.

CSAPO, A., The mechanism of myometrial function and its disorders, in BOWES, K. (ed.) Modern Trends in Obstetrics and Gynæcology (Second Series) London. Butterworth & Co. 1955. 20—49.

COURTICE, F C., The blood volume of normal animals. *J Physiol.* (Lond.) 1943. *102* 290—305.

FUCHS, F The red cell volume of the maternal and foetal circuits of the guinea pig placenta. *Acta physiol. scand.* 1953. *28* 162—171

FUCHS, F Studies on the passage of phosphate between mother and foetus in the guinea pig. Copenhagen, E. Munksgaard 1957 pp. 127

FUCHS, F and A.-R. FUCHS, Studies on the placental transfer of phosphate in the guinea pig I The transfer from mother to foetus. *Acta physiol. scand.* 1957 a. *38* 379—390.

FUCHS, F and A. R. FUCHS, Studies on the placental transfer of phosphate in the guinea pig II. The transfer from foetus to mother *Acta physiol. scand* 1957 b. *38.* 391—397

Fuchs, F. and A. R. Fuchs, Studies on the placental transfer of phosphate in the guinea pig. III. Phosphorus compounds in the placenta and their uptake of radioactive phosphorus. *Acta physiol. scand.* 1957. 39. 277—283.

Fuchs, F. and A. R. Fuchs, Induction and inhibition of labour in the rabbit. *Acta endocr.* (Kbh.) 1958. 29. 615—624.

Fuchs, F. and A.-R. Fuchs, Foetal optagelse af fosfat ved forlænget graviditet hos kaniner. *Förh. Nord. Fören. Obstet. Gynec. 11. Congr.* Helsinki 1960. (In print)

Hevesy, G. and L. Hahn, Rate of renewal of the acid soluble organic phosphorus compounds in the organs and the blood of the rabbit. *Kgl. Danske Videnskab. Selsk. Biol. medd.* 1940. 15. 7 : 1.

Acta physiol. scand. 1961. 52. 75—89

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Influence of Local Temperature Changes in the Preoptic Area and Rostral Hypothalamus on the Regulation of Food and Water Intake

By

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Abstract

Andersson, B. and B. Larsson. *Influence of local temperature changes in the preoptic area and rostral hypothalamus on the regulation of food and water intake.* Acta physiol. scand. 1961. 5. 75—89. — Local cooling of the preoptic area and rostral hypothalamus induced eating in the fed goat. After dehydration to the state of aphagia, cooling of this area inhibited the animal's urge to drink and induced eating. Although in a normal goat feeding practically stops when the rectal temperature exceeds 40° C (Appleman and Delouche 1958), local cooling of the preoptic area and the rostral hypothalamus induced the goat to eat hay at body temperatures above 41° C. Warming the same area inhibited eating in the hungry animal and induced the goat to drink large quantities of water.

After inactivation of the preoptic "heat loss centre" by proton irradiation in a goat, the animal became adipsic but continued to eat hay with seemingly good appetite at body temperatures above 41° C. The anorexic effect of warming the preoptic area thus does not seem to be due to a direct thermal effect on the hypothalamic "appetite centre". The results provide direct evidence in favour of Brobeck's (1948) "thermostatic" theory of the regulation of food intake and justify the further extension of this theory to involve also the regulation of water intake.

Several investigations have indicated that food intake is regulated through the interaction of a "satiety centre" in the ventromedial hypothalamus and an "appetite centre" in the lateral hypothalamus (Brobeck 1955). Water intake



Fig. 1. X-ray pictures of the head of the "thermode" goat taken from the side ( ) and from above (b).
T. The body of the thermode implanted medially in the preoptic area and rostral hypothalamus.
A. The needle applicator used for measurements of brain temperature close to the surface of the thermode.
s. Dental root screws used for fixation of dental cement to the skull bone.

seems to be regulated from parts of the hypothalamus partly overlapping the "satiety centre" and located medially and somewhat rostral to the "appetite centre" (ANDERSSON and McCANN 1955). Cellular dehydration has been considered the main factor stimulating the hypothalamic "drinking centre" (VOLF 1950, ANDERSSON 1952). The changes in the internal environment of importance for the regulation of food intake may however be multiple. A theory of a glucostatic mechanism regulating food intake has been introduced (MAYER 1952) but adjustments of feeding may also be made in relation to the amount of stored fat in the body (KENNEDY 1953). BROBECK (1948, 1960) has stressed the intimate correlation between body temperature and food intake and has presented a "thermostatic" theory. He suggests that in inhibiting food intake heat acts either upon heat sensitive neurons of the rostral hypothalamus and the preoptic area or directly upon neurons of the "appetite centre".

The experiments reported here provide evidence in favour of BROBECK's suggestion that a rise of the body temperature may inhibit food intake by the influence of heat sensitive neurons in the preoptic area and rostral hypothalamus. They also indicate that the hypothalamic "satiety centre" may be inhibited by the influence of cold sensitive elements in the same area and further that thermosensitive neurons in this part of the brain influence water intake in the reverse manner that they influenced food intake.

A further study of the effect on food and water intake and on temperature-regulation of lesions of various size in the preoptic area will be presented in a following publication (ANDERSSON, LARSSON and REHED 1961).

Fig. 2. *Drawings of a medial sagittal section through the hypothalamus ( ) and of a transverse section through the preoptic area of the goat (b) to show the positions of the thermode (T) and the needle applicator (A).*

ac Commissura anterior
Cd Nucleus caudatus
cfd Columna fornicis descendens
Ch Chiasma opticum
CI Capsula interna
CM Corpus mamillare
Ep Epiphysis
MI Massa intermedia
N Neurohypophysis
PA Adenohypophysis
3 Third ventricle

Methods

The experiments were made in two goats. One animal had a silver thermode implanted medially in the preoptic area and rostral hypothalamus and the other goat had had its preoptic "heat loss centre" destroyed by proton irradiation. The principles for the use of the high-energy proton beam as a neurosurgical tool was described earlier (Larsson *et al.* 1958, Leksell *et al.* 1960). In the present animal permanent platinum iridium electrodes, previously implanted into the "heat loss centre" had been used as points of aim for the irradiation. This technique will be described elsewhere (Larsson and Andersson 1961).

Technique for implantation of a silver thermode into the brain stem. The technique used was similar to that previously described for implantation of permanent electrodes into the hypothalamus of horned goats (Andersson, Persson and Ström 1960). With the animal generally anesthetized, a holder with rods for guidance of the thermode and needle applicator for brain temperature recording was temporarily screwed onto the surface of the skull bone. The correct placement of the holder was facilitated by previous X-ray studies and measurements on the skull. An opening in the skull bone, fitting the thermode, was then made along the midline and a smaller hole for the needle applicator was drilled through the bone more laterally. The dura mater was split along the midline of the brain to allow the introduction of the thermode into the brain. When the thermode and the needle applicator had been placed in the positions shown in Fig. 1 and 2, they

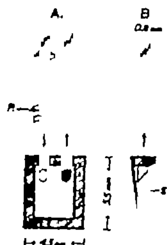


Fig 3. Drawings of the silver thermode used for cooling and warming of the preoptic area and the rostral hypothalamus.
A: Lateral view
B Frontal view
P Polethylene tubing used as insulation of the inwards leading cannula.
S: The side walls of the thermode consisting of 0.2 mm thick silver plates.

were permanently attached to the bone of the skull with dental cement filling the holes in the bone. The dental cement was further secured to the bone by the help of dental root screws. The holder used for temporary fixation and guidance of the thermode was then removed, leaving only the two cannulas of the thermode and the base of the needle applicator sticking up on the dorsal surface of the skull. The skin incision was sutured round the protruding layer of dental cement. Polyethylene tubing connected to the cannulas of the thermode and the leads from the needle applicator were fixed to one horn of the animal and the whole equipment was protected from external damage in the manner previously described (ANDERSSON *et al* 1960 a) The kind of thermode used is shown in Fig 3.

Technique of central cooling When used for cooling the thermode was perfused via a 1.5 m long rubber tubing connecting a pressure flask, containing iced water with the polyethylene tubing attached to the inwards leading isolated cannula of the thermode. The end of the rubber tubing was hereby fixed on one horn. An applicator (Ellab type KC41) was placed in the end of the rubber tubing allowing measurements of the temperature of the perfusion water at the junction of the rubber tubing and the polyethylene tubing on the horn. Since the animal was kept in its usual environment (collared in a metabolism cage) during all experiments, it was necessary to have a lead of this length to allow it full freedom to move as it was used to. The long rubber tubing however had the disadvantage that the temperature of the perfusion water entering the polyethylene tubing was as high as 19 to 20° C when the perfusion was performed in the usual environmental temperature of the goat (22° C) Perfusions made in the above described manner lowered the temperature at the surface of the thermode 9 to 10° C. The latency time from beginning of perfusion with cold water to maximal lowering of the brain temperature at the surface of the thermode was of the order of 50 secs to one minute. When the perfusion was suddenly stopped, it took one to one and a half minute for the temperature close to the surface of the thermode to reach blood temperature level again.

The cooling experiments reported here were all performed in the manner just described. However by using shorter tubing connecting the pressure flask with the thermode and by having the goat placed in a colder environment it was possible in other experiments to obtain a more intense central cooling with principally the same technique

Technique of central warming All experiments involving central warming were also performed in an external temperature of 22° C. To obtain a reasonably high temperature

of the perfusion water although using a long lead, an extra outlet for the warm water had to be fitted to the rubber tubing close to the head of the goat. Hot tap water (65 C) was used for perfusion and the perfusion pressure was kept close to 200 mm Hg Due to heat losses in the rubber tubing, the temperature of the water at the junction on the horn was only of the order of 55 C. Identical perfusion technique was used in all experiments and was found to cause temperature rise of about 8° C at the external surface of the thermode. The latency time from start of perfusion with warm water until the temperature at the surface of the thermode reached its maximum was about one minute. Thirty secs after a sudden stop of the perfusion, the temperature t the surface of the thermode had again fallen to the level of blood temperature.

Temperature recordings: Brain temperature at the surface of the thermode was recorded by connecting the leads from the implanted needle applicator ("Ellab" type K 8) to an "Ellab" Electric Universal Thermometer (type TE 3)

Blood temperature was recorded in a similar manner using an Ellab" applicator (type F 6) inserted through the jugular vein into the superior vena cava at a distance of 5 to 10 cm from the heart.

Ear surface temperature, as an index of peripheral vasodilatation and vasoconstriction, was measured by the help of an "Ellab" surface applicator (type E 5) attached to the dorsal surface of one ear near its tip.

Rectal temperature was measured occasionally during the experiments using mercury thermometer

At times when the thermode was not perfused, no or very small differences were observed between blood and brain temperature. Rectal temperature, however was found to be poor index of real body temperature, since it lagged considerably behind relatively rapid changes in blood and brain temperature.

Recordings of ruminal motility The animals were supplied with closed ruminal fistulas and ruminal motility was occasionally recorded as previously described (ANDERSSON KITCHELL and PERSSON 1959)

Recordings of respiratory rate were made as previously described (ANDERSSON PERSSON and STROM 1960 b)

Care of the animals. The goats were constantly kept collared in metabolism cages and had for more than month after the operations become used to the experimental conditions. They were placed in room where the temperature was kept at 22 C and were fed regularly at 9 o clock a. m. They then obtained 250 g groats with 4 g N Cl added and 2 kg hay The groats was usually consumed during 15 to 20 minutes whereas the hay ration lasted till in the afternoon. Some less palatable residuals of the hay ration were usually left on the bottom of the foddering-rack till next feeding time and were then discarded.

All observations concerning food intake reported here concern the animals consumption of hay

With the exception of certain periods of purposely induced water deprivation, the goats had always free access to water The water container was placed t the side of the foddering rack in front of the goats. Daily water intake and urinary output were recorded.

Results

Localization of the thermode and approximate degree and extent of central cooling and warming

Since the thermode" goat is still alive and is used for further experiments, no histological localization of th site of the thermode has as yet been made.

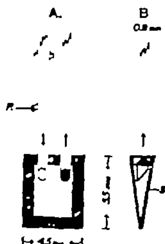


Fig. 3. Drawings of the silver thermode used for cooling and warming of the preoptic area and the rostral hypothalamus.
A: Lateral view
B: Frontal view
P Polethylene tubing used as insulation of the inwards leading cannula.
S The side walls of the thermode consisting of 0.2 mm thick silver plates.

were permanently attached to the bone of the skull with dental cement filling the holes in the bone. The dental cement was further secured to the bone by the help of dental root screws. The holder used for temporary fixation and guidance of the thermode was then removed, leaving only the two cannulas of the thermode and the base of the needle applicator sticking up on the dorsal surface of the skull. The skin incision was sutured round the protruding layer of dental cement. Polyethylene tubing connected to the cannulas of the thermode and the leads from the needle applicator were fixed to one horn of the animal and the whole equipment was protected from external damage in the manner previously described (ANDERSSON *et al.* 1960 a). The kind of thermode used is shown in Fig. 3.

Technique of central cooling. When used for cooling the thermode was perfused via a 1.5 m long rubber tubing connecting a pressure flask, containing iced water with the polyethylene tubing attached to the inwards leading, isolated cannula of the thermode. The end of the rubber tubing was hereby fixed on one horn. An applicator (Ellab type KC#1) was placed in the end of the rubber tubing allowing measurements of the temperature of the perfusion water at the junction of the rubber tubing and the polyethylene tubing on the horn. Since the animal was kept in its usual environment (collared in a metabolism cage) during all experiments, it was necessary to have a lead of this length to allow it full freedom to move as it was used to. The long rubber tubing, however had the disadvantage that the temperature of the perfusion water entering the polyethylene tubing was as high as 19 to 20° C when the perfusion was performed in the usual environmental temperature of the goat (22° C). Perfusions made in the above described manner lowered the temperature at the surface of the thermode 9 to 10° C. The latency time from beginning of perfusion with cold water to maximal lowering of the brain temperature at the surface of the thermode was of the order of 50 secs to one minute. When the perfusion was suddenly stopped, it took one to one and a half minute for the temperature close to the surface of the thermode to reach blood temperature level again.

The cooling experiments reported here were all performed in the manner just described. However by using shorter tubing connecting the pressure flask with the thermode and by having the goat placed in colder environment, it was possible in other experiments to obtain a more intense, central cooling with principally the same technique.

Technique of central warming. All experiments involving central warming were also performed in an external temperature of 22° C. To obtain a reasonably high temperature

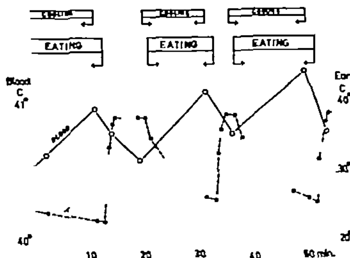


Fig. 4. *The second part of an experiment in which the preoptic area and rostral hypothalamus was repeatedly cooled.*

The goat had free access to hay during the experiment which started at normal feeding time. The increasing blood temperature during the periods of central cooling was due to shivering and peripheral vasoconstriction. During the intervals between periods of central cooling, heat loss mechanisms were mobilized, which worked to put the body temperature back to a normal level The animal was eating hay with seemingly good appetite during the periods of central cooling, but stopped eating simultaneously to the onset of peripheral modulatation (rise of ear temperature) following upon discontinuation of central cooling Towards the end of the experiment central cooling induced eating in spite of blood temperature well abov 41 C.

The non-fed animal with free access to water APPLEMAN and DELOUCHE (1958) found in the normal goat that feeding practically stops when rectal temperature exceeds 40° C. It therefore seemed to be of interest to find out whether or not the present animal would eat hay at still higher body temperatures when its preoptic area and rostral hypothalamus was cooled, and further to try to determine the body temperature at which the goat would stop eating when no longer centrally cooled. These experiments started at regular feeding time. The second part of such an experiment is shown in Fig 4 The rectal temperature was 39.2 C and the blood and brain temperature 39 6° C when this experiment started. During the first 10 min of central cooling the goat had no access to hay Cooling of the preoptic area and rostral hypothalamus was then found to increase the strength and the frequency of ruminal contractions. When later given hay the animal started to eat with a good appetite. During the first interruption of central cooling the goat continued to eat, but with diminished intensity The blood temperature was then 40.2 C. During th second interruption of central cooling when blood temperature had reached 40 6° C, the goat stopped eating but started again during the following period of central cooling As is shown in

Fig 4 intense eating of hay could still be induced by cooling the preoptic area and rostral hypothalamus at blood temperatures above 41.2 C.

Eating generally started 30 sec to 1 1/2 min after commencement of cooling and continued for one to two minutes after cessation of cooling Eating was then seen to stop simultaneously to the onset of peripheral vasodilatation (Fig. 4)

The water deprivated goat with free access to food. On two occasions the goat was not given any water for three days but was fed its usual ration. On both occasions it stopped eating on the third day of water deprivation and was then very eager to drink minute amounts of water offered. Two or three minutes after beginning of central cooling however it no longer showed any interest in offered water but instead started to eat accessible hay One minute after the cooling was stopped the goat turned its attention from the hay and started to drink large amounts of water from the water container which had been filled during the period of central cooling

c) *Other effects of cooling the preoptic area and rostral hypothalamus*

During the 24 hours following experiments in which the preoptic area and rostral hypothalamus had been cooled for longer periods the goat was found to have passed large amounts of diluted urine and to have drunk about three times the normal amount of water This phenomenon will be the subject of further studies.

Effects of warming the preoptic area and rostral hypothalamus

a) *Thermoregulatory effects*

As was expected from the results of earlier investigations (MAGOUN *et al.* 1938 and others) warming the preoptic area and rostral hypothalamus had the reverse thermoregulatory effect to that of cooling The perfusion of the thermode with warm water causing a rise of the temperature at its external surface to 48 to 49 C, resulted in polypnea and peripheral vasodilatation (Fig. 5) and thus led to a fall of the body temperature.

b) *Alimentary effects*

The experiments in which local warming of the preoptic area and the rostral hypothalamus was performed, started at regular feeding time when the goat had just obtained its hay ration and had started to eat with a good appetite. On most occasions the animal had free access to water having the filled water container placed in the usual manner Thirty secs to one minute after commencement of central warming the goat stopped eating The brain temperature close to the surface of the thermode was then of the order of 45 to 46 C. Within a minute after the animal had stopped eating it showed obvious signs of intense thirst. If no water was accessible it started to lick the drops of water coming out of the outlet tubing of the thermode, but no longer showed any interest in

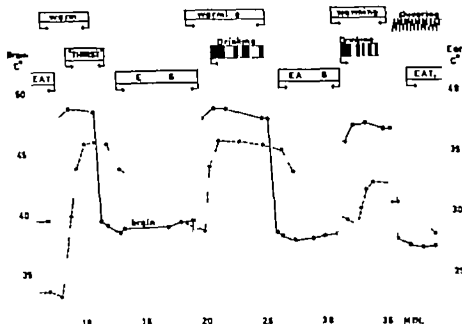


Fig. 5. *Results of warming the preoptic area and rostral hypothalamus in the previously hungry animal.*
Brain temperature was recorded close to the surface of the thermode. The goat was fed hay at the beginning of the experiment and had free access to water except during the first period of central warming. During the periods of warming eating stopped simultaneously to the onset of peripheral vasodilatation (rise of ear temperature), and started again when ear surface temperature had begun to fall after discontinuation of central warming. The perfusion of the thermode with warm water induced a strong urge to drink. During the first period of central warming, when the water container was temporarily removed, it was evidenced by the animal's licking of the drops of water coming out of the outlet tubing of the thermode ("thirst") and later on by the repeated drinking of large amounts of water during the periods of central warming.

water a minute after central warming was discontinued. If water was accessible during warming of the preoptic area and rostral hypothalamus the goat turned to the water container and started to drink. It continued to drink large amounts of water in one sequence. If central warming was not interrupted, the goat, after the first long period of drinking, continued to hold its head over the water container and now and then drank again for shorter periods of time (Fig. 5). During intervals between periods of central warming the goat, however, refused to drink. Fig. 5 shows the time sequence of the periods of eating and drinking during and between repeated periods of central warming. In this experiment the goat drank 3.9 l of water at 18° C due to warming of the preoptic area and rostral hypothalamus. As can be seen in the figure there was a close correlation in time between stopping eating, the onset of peripheral vasodilatation and the start of drinking. The repeated mobilization of heat loss mechanisms and the drinking of large amounts of relatively cold water gradually lowered the body



Fig. 6. *An X-ray picture of the head of the goat which had had its preoptic "heat loss centre" permanently inactivated by proton irradiation.*
The tips of the two rostral electrodes were implanted into the dorsal part of the "heat loss centre". The encircled area marks the approximate sagittal extent of the irradiated area. The tip of the posterior electrode (C) was situated outside the irradiated area. Electrical stimulation via this electrode caused shivering. Prolonged stimulation resulted in considerable rise of body temperature (Fig. 7).

temperature of the goat more than 2° C which, towards the end of the experiment, resulted in shivering during the intervals between periods of central warming

Experiments in a goat in which the preoptic "heat loss centre" was permanently inactivated

In a series of goats, having permanent electrodes implanted in the preoptic "heat loss centre" the tips of the electrodes were used as points of aim for proton irradiation of this centre (LARSSON and ANDERSSON 1961) The effect of the subsequent lesions on food and water intake and on temperature regulation was studied and will be described elsewhere (ANDERSSON *et al.* 1961) Three animals belonging to this series developed permanent adipsia, in two the adipsia was accompanied by hypophagia or aphagia. The third goat, however retained its urge to eat to an almost normal extent. This goat had one electrode placed slightly dorsal and posterior to the irradiated part of the brain stem (Fig 6 C) Electrical stimulation via this electrode caused shivering and by continuing the stimulation the body temperature of the animal could be increased more than 2° C. The complete inactivation of the "heat loss centre" was evidenced by the fact that the animal no longer reacted with peripheral vasodilatation and polypnea during hyperthermia. Stopping stimulation when the animal's body temperature had reached 41.5 C was thus not followed by any increase in the ear surface temperature or any increased respiratory rate (Fig 7) Of special interest in this connection was the observation that the goat retained its urge to eat hay with a seemingly good appetite even at this very high level of body temperature (Fig 7)

No histological examination of the preoptic lesion has so far been made in

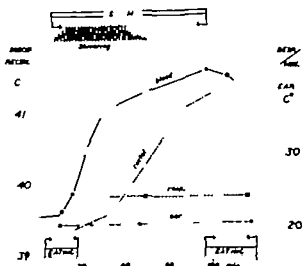


Fig. 7 *Results of electrical stimulation via electrode C in the goat which had had its preoptic "heat loss centre" permanently inactivated.* The prolonged stimulation (stim.) caused shivering which led to 2° C increase in body temperature. The total absence of central regulation against hyperthermia was evidenced by the lack of polypnea (low respiratory rate) and by remaining peripheral vasoconstriction (unchanged ear surface temperature) after discontinuation of the stimulation. During the periods marked "eating" the animal was offered hay and was eating with good appetite. During the second period it was eating as intensively as before in spite of body temperature well above 41° C.

this animal, but the extent of the area subjected to irradiation is indicated in Fig 6. The irradiation did not involve any part of the hypothalamic "feeding centre"

Even in another respect the regulation of food intake was in the irradiated animal different from that of a normal goat. To test the completeness of its adipsia the goat was on three occasions not given water via the ruminal fistula for three or four days. Unlike a normal goat it continued to eat its daily ration of food even on the fourth day of water deprivation. The degree of dehydration was then very severe (Plasma Na 174 meq/l Plasma Cl 134 meq/l)

Discussion

The present series of experiments was started because it had been observed that lesions in the preoptic area, intentionally made to destroy the "heat loss centre" caused permanent adipsia and in cases with larger lesions in addition hypophagia or even aphagia (ANDERSSON *et al.* 1961) These observations led to the assumption that the central projections from the hypothalamic "feeding" and "drinking centres" pass via the preoptic area to the rhinencephalon and that these connections are essential for the development of a conscious urge to eat and to drink. The first experiment in which alimentary effects of central cooling were studied, was therefore performed as an attempt to cause a [illegible] block of the central projections from the hypothalamic drinking [illegible] in the thirsty goat. For this reason the animal had been deprivated of water for

three days and was no longer eating the food offered. Cooling was found to inhibit the animal's urge to drink but in addition it also induced eating. This finding indicated a close interaction between thermosensitive elements in the preoptic area and rostral hypothalamus and the hypothalamic "feeding centre" as suggested by BROBECK (1960). The animal, being "basically hungry" might however have started to eat because it was released from the uncomfortable sensation of strong thirst. Similar experiments were therefore made when the goat had been kept with free access to water. The experiments were also extended to studies of alimentary effects due to local warming of the preoptic area and the rostral hypothalamus. It was found that central cooling induced eating in the fed goat, also, and that the animal could be induced to eat hay with a seemingly good appetite in spite of a body temperature much higher than that at which a normal goat stops eating (APPLEMAN and DELOUCHE 1958). Local warming of the preoptic area and the rostral hypothalamus had the reverse effect. It inhibited eating in the previously hungry animal and induced it to drink large quantities of water although not having shown any signs of thirst the moment before local warming started.

The short latencies of the alimentary effects of central cooling and warming and the reversibility of these effects makes it unlikely that they should be due to chemical changes of the internal environment which have a direct stimulatory or inhibitory effect on the hypothalamic "feeding" and "drinking centres". It seems more probable that the changed activity in these "centres" may have been due to a neural influence. The close time relation between the start of eating and the onset of peripheral vasoconstriction and between stop of eating and the onset of peripheral vasodilatation is in evidence of an intimate functional correlation between a thermoregulatory mechanism and the hypothalamic "feeding centre". This is entirely in line with BROBECK's "thermostatic" theory as illustrated by the following quotation from his recent review "Food and Temperature" (1960):

"The possibility that the necessary brake (satiety) is applied by the heat, itself, was proposed first by BOOTH and STRANG (1936) after observations had been made on normal and obese human subjects. They recorded the elevation of skin temperature which follows a meal and attempted to correlate this elevation with the onset of satiety. In their view the body appreciates the sensation of warmth in the skin, and this leads in turn to a feeling of comfort and satiation. It now appears more likely that the appreciation is not peripheral, but central — that the SDA (specific dynamic action) acts directly upon cells in or just ahead of the hypothalamus to evoke cutaneous vasodilatation, and that this is accompanied by central inhibition of appetite and induction of satiety."

Since MAGOUN *et al.* (1938) had shown that local warming of the preoptic area causes the mobilization of various heat loss mechanisms, this part of the brain has been considered to be the site of a "heat loss centre" which is exclusively concerned with the regulation against hyperthermia. More recent

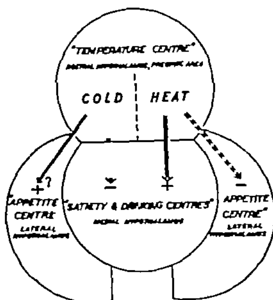


Fig. 8. *A tentative explanation of the alimentary effects of cooling and warming the preoptic area and rostral hypothalamus.*
The suggested explanation of the effect of warming on food intake is in accordance with Brobeck's (1948, 1960) "thermostatic theory"

studies have however shown that local cooling within (Kundt, Brück and Hensel 1957, Andersson 1961) and electrical stimulation in the close vicinity of (Andersson 1957) the same area induce shivering and peripheral vasoconstriction. The latter findings indicate that this part of the brain is not only the site of a heat loss mechanism, but is also involved in the defense against hypothermia.

Since cooling of the preoptic area and the rostral hypothalamus induced eating in the fed, normothermic animal, it may be assumed that the stimulation of a pre- and supraoptic cold defense mechanism activates the hypothalamic "appetite centre" either directly or by inhibiting the ventromedial "satiety centre". The latter may be the most probable explanation since cooling at the same time inhibited the animal's urge to drink and the "drinking centre" is anatomically closely related to the "satiety centre" (Stevenson, Welt and Orloff 1950, Andersson and McCann 1955).

Cellular dehydration has been considered to be the main factor eliciting the sensation of thirst (Wolf 1950, 1958). But the strong urge to drink, induced in the "thermode" animal by warming the preoptic area and the rostral hypothalamus is not likely to have involved any change in the osmolarity of the internal environment. The urge to drink disappeared soon after discontinuation of central warming and rapidly returned when central warming was started again (Fig. 5). It may therefore be easier to explain the drinking effect as due to a stimulatory action exerted from the preoptic "heat loss centre" on a hypothalamic mechanism concerned with the regulation of water intake. To judge from the present findings, the activation of a central mechanism concerned

with the regulation against hypothermia, would have the reverse effect on the hypothalamic "drinking centre" In summary the results reported here may thus be tentatively explained in the manner shown in Fig 8.

One main objection to this explanation is that the cooling and the warming of the preoptic area and the rostral hypothalamus may have affected directly the areas of the hypothalamus concerned with the regulation of food and water intake. A spread of the local warming and cooling to the lateral "appetite centre" seems unlikely however In addition, the goat which had had the preoptic "heat loss centre" destroyed by proton irradiation, did eat at body temperatures well above 41 C. This seems to exclude the possibility that local warming has any direct inhibitory effect on the activity of the hypothalamic "appetite centre" Presently however it can not be ruled out that the perfusion of the thermode with warm and cold water may have changed the temperature in the "satiety" and "drinking centres" especially since the latter was found to extend into the rostral hypothalamus (ANDERSSON and MCCANN 1955) Provided the local warming acted as a non-specific stimulus to the neurons and the local cooling directly inhibited all neuronal activity within the affected area of the brain, the observed alimentary effects could be explained as being due to a spread of heat and cold to the "satiety" and "drinking centres" Such an explanation seems unlikely since the perfusion of the thermode with warm water was only seen to induce thermoregulatory and alimentary effects. Had the local warming acted as a non-specific stimulus to all neurons within the heated area, it probably would have caused a much more complex and diffuse reaction in the animal.

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References

ANDERSSON, B. Polydipsia caused by intrahypothalamic injections of hypertonic NaCl-solutions. *Experientia* (Basel) 1952. VIII. 157—158.

ANDERSSON, B Cold defense reactions elicited by electrical stimulation within the septal area of the brain. *Acta physiol. scand.* 1957 *41* 90—100

ANDERSSON, B., T be published. 1961

ANDERSSON, B., R. KITCHELL and N PERSSON A study of rumination induced by milking in the goat. *Acta physiol. scand.* 1958. *44* 92—102.

ANDERSSON B., B. LARSSON and B. REXED, T be published. 1961

ANDERSSON B. and S. M. MCCANN, Drinking, antidiuresis and milk ejection from electrical stimulation within the hypothalamus of the goat. *Acta physiol. scand.* 1955. 35. 191

ANDERSSON B., N PERSSON and L. STRÖM, Functional localization as an aid to the
of permanent electrodes into the hypothalamus of horned goats. *Acta physiol.*
50. 49—53.

ANDERSSON, B., N. PERSSON and L. STRÖM, Post-stimulatory electrical
"heat loss centre" concomitant with persistent thermoregulatory
1960 b. 50. 54—61

APPLEMAN, R. D. and J. C. DELOUCHE, Behavioral, physiological and biochemical responses of goats to temperature 0° to 40° C. *J. Anim. Sci.* 1958. *17* 326—335.

BOOTH, G. and J. M. STRANG, Changes in temperature of skin following ingestion of food. *A.M.A. Arch. intern. Med.* 1936. *57* 533—543.

BROBECK, J. R., Food intake as a mechanism of temperature regulation. *Yale J. Biol. Med.* 1948. *20.* 545—552.

BROBECK, J. R., Neural regulation of food intake. *Ann. N. Y. Acad. Sci.* 1955. *63.* 44—55

BROBECK, J. R., Food and temperature. *Rec. Progr. Horm. Res.* 1960. *16.* 439—466

KENNEDY, G. C., The role of depot fat in the hypothalamic control of food intake in the rat. *Proc. roy. Soc. B.* 1953. *140.* 578—592.

KUNDT, H. W., K. BRÜCK and H. HENSEL, Das Verhalten der Hautdurchblutung bei Kühlung des vorderen Hypothalamus. *Naturwissenschaften* 1957 *44* 496—497

LARSSON, B. and B. ANDERSSON, To be published. 1961

LARSSON, B., L. LEKSELL, B. REXED, P. SOURANDER, W. MAIR and B. ANDERSSON, The high-energy proton beam as a neurosurgical tool. *Nature* (Lond.) 1958. *182.* 1222.

LEKSELL, L., B. LARSSON, B. ANDERSSON, B. REXED, P. SOURANDER and W. MAIR, Lesions in the depth of the brain produced by a beam of high energy protons. *Acta radiol.* (Stockh.) 1960. *54* 251—264.

MAGOUN, H. W., F. HARRISON, J. R. BROBECK and S. W. RANSON, Activation of heat loss mechanisms by local heating of the brain. *J. Neurophysiol.* 1938. *1* 101—114.

MAYER, J., The glucostatic theory of regulation of food intake and the problem of obesity. *Bull. New Engl. med. Cent.* 1952. *14* 43—49

STEVENSON, J. A. F., L. G. WELT and J. ORLOFF, Abnormalities of water and electrolyte metabolism in rats with hypothalamic lesions. *Amer. J. Physiol.* 1950 *161* 35—39.

WOLF, A. V., Osmometric analysis of thirst in man and dog. *Amer. J. Physiol.* 1950. *161* 75—86.

WOLF, A. V., Thirst. Physiology of the urge to drink and problems of water lack. *Springfield Ill. Charles C. Thomas.* 1958.

Acta physiol. scand. 1961. 52. 90—98

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A Criticism of the Carcass Analysis Procedure for the Determination of Amino Acid Requirements

By

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Abstract

ERICSON, L. E. *A criticism of the carcass analysis procedure for the determination of amino acid requirements.* Acta physiol. scand. 1961. 52. 90—98. — The carcass analysis procedure is examined as a method for the determination of amino acid requirements. Using a simple reaction sequence to illustrate the fate of an amino acid in the body, enzyme kinetics are applied to demonstrate the impossibility of estimating the requirement of one compound in relation to another from knowledge of only the concentrations of the two compounds in the organism. The further complications arising from (a) the known individual variations in the concentrations of enzymes in the body, (b) the utilization by the organism of certain essential amino acids for purposes other than protein synthesis, (c) the partial ability of the organism to synthesize some essential amino acids, (d) the different rates of breakdown and excretion of the amino acids and (e) the fact that the body consists not of one but of a large number of different proteins with varying metabolic stability are briefly discussed. Some experimental data are cited which indicate that the carcass analysis procedure can give requirement values that are greatly erroneous. The criticism is presented with the hope that it will stimulate further theoretical and experimental studies in order to define the limitations and possibilities of the method.

In discussions concerning the amino acid requirements of higher animals the opinion is often expressed that these requirements are reflected by the amino acid composition of the animal under study (BEACH, MUNKS and ROBINSON 1943, MUNKS *et al.* 1945, BOCOBO *et al.* 1952, WILLIAMS *et al.* 1954, SPEAR

PERNAK 1958, ALBANESE 1955 1959 MITCHELL 1959) It is stated, that if the requirement of *one* amino acid is known, the body's need for all the other amino acids can be calculated from its amino acid composition. In other words, the *ratios of the requirements* of the different essential amino acids should be identical with the *ratios of the concentrations* of these amino acids in the animal body This method of determining the amino acid requirements from body composition data is generally referred to as the carcass analysis procedure.

Various animal species have a remarkably similar amino acid composition (*cf* BEACH, MUNKS and ROBINSON 1943 BOCONO *et al.* 1952, WILLIAMS *et al.* 1954 MITCHELL 1959) This would make the caracass analysis procedure more useful as the amino acid composition of one kind of animal *e. g.* the rat, could be used for calculating the amino acid requirements of another kind of animal, *e. g.* the human. It is also postulated, that since muscle protein represents nearly three quarters of the total protein of higher animals it should be sufficient to know the amino acid composition of this tissue in order to estimate the desirable ratios of dietary amino acids. This approximation seems to be supported by the isotope experiments of SCHÖNHEIMER *et al.* (1949) and others who have shown, that of the total quantity of protein in the body that is exchanged per unit time, muscle protein provides about sixty five per cent. Similarly when protein is lost from the body during a period of protein starvation, most of it is derived from muscle tissue (ADDIS, POO and LEW 1936) It may therefore be justifiable to consider as some authors have done (*cf* ALBANESE 1955 1959 SHARPENAK 1958, MITCHELL 1959) only the composition of the muscle protein when estimating amino acid requirements by the carcass analysis method. What may not be correct, however is the basic assumption that the composition of a tissue permits a prediction of the need for maintenance or growth of this tissue.

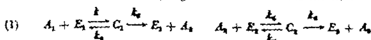
Simplified enzyme kinetic consideration

All organic constituents of the food undergo series of reactions in the organism before being excreted. A simple reaction sequence that could illustrate this fact and serve as a model for enzyme kinetic considerations, would be one in which compound (1) is first in one step converted to the form in which it is normally found in the cell (2) and thereafter in one further step metabolized to the form (3) in which it is excreted



$$(1) \rightarrow (2) \rightarrow (3)$$

It is assumed here that in these irreversible convertions no other substance participates, part from two enzymes catalyzing the reactions. We can therefore write



$$(1)\qquad A_1 + E_1 \underset{k_2}{\overset{k}{\rightleftharpoons}} C_1 \xrightarrow{k_3} E_1 + A_2 \qquad A_2 + E_2 \underset{k_5}{\overset{k_4}{\rightleftharpoons}} C_2 \xrightarrow{k_6} E_2 + A_3$$

where

The difficulty in finding the correct interpretation for data obtained in isotope studies of the type performed by Schonheimer and others should be mentioned.

A = the form in which the compound is supplied.
A_2 = the form in which the compound appears in the cell after having been transformed by the enzyme E_1
A_3 = the form in which the compound is excreted after having been further converted by the enzyme E_2.
C_1 and C_2 = the enzyme-substrate complexes formed when A reacts with E_1 and A_2 with E_2.
k_1—k_6 = rate constants.

Letting the symbols represent both the type of compound and the concentration (REINER 1959) we can write the rate equations for A_1, C_1, A_2, C_2 and A_3

(2) $$\frac{dA}{dt} = -k_1 A_1 E_1 + k C_1$$

(3) $$\frac{dC_1}{dt} = k_1 A_1 E_1 - k_2 C_1 - k_3 C_1$$

(4) $$\frac{dA_2}{dt} = k_3 C_1 - k A_2 E_2 + k_5 C_2$$

(5) $$\frac{dC_2}{dt} = k A_2 E_2 - k_5 C_2 - k_6 C_2$$

(6) $$\frac{dA_3}{dt} = k_6 C_2$$

The total amount of the first enzyme will be called E_{t1} and the total amount of the second E_{t2}. The two enzymes appear both in free form and in the form of enzyme-substrate complexes, which gives the conservation equations

(7) $$E_{t1} = E_1 + C_1 \text{ and}$$

(8) $$E_{t2} = E_2 + C_2$$

The situation that in the present discussion is of most interest, arises when the concentration of A_2 does not vary with time the composition of the animal must not change from one moment to the next. This means that $\frac{dA_2}{dt} = 0$ or in other words, that the rate of formation of A_2 is equal to the rate of disappearance. Assuming steady state conditions for the two enzyme-substrate complexes C_1 and C_2 it can be shown that

(9) $$\frac{k_3 E_{t1} A}{K + A} = \frac{k_6 E_{t2} A_2}{K + A_2}$$

where

(10) $$K = \frac{k_2 + k_3}{k_1} \text{ and}$$

(11) $$K = \frac{k_5 + k_6}{k}$$

If this constant concentration of A is called A_{ss} and, by rearrangement of equation (9) expressed as a function A_1 we obtain

(12) $$A_{ss} = \frac{k_6 E_{t2} K A_2}{k_3 E_{t1} K + (k_6 E_{t2} - k_3 E_{t1}) A_2}$$

The products $k_6 E_{t1}$ and $k_4 E_{t2}$ correspond to the maximal rates of formation of A_4 and A_{3e}, *e. g.* the rates obtained when C_1 and C_2 have their maximal values. This occurs when the enzymes are saturated with substrate (E_1 and $E_2 = 0$ in equation (7) and (8)). If these maximal rates are called V and V equation (12) can be written in a slightly more condensed form

$$(13) \qquad A_{se} = \frac{V \; K_2 \; A_1}{V \; K_1 + (V_2 - V) \; A_1}$$

It can be seen, that the relation between A_{se} and A is not straightforward. If A is small, A_{se} is approximately proportional to A_1 but as A_1 increases towards greater values, A_{se} gradually becomes independent of A_1. The value of A_{se} is furthermore dependent on a number of velocity constants and also on the concentrations of the enzymes E_1 and E_2. The last fact is especially noteworthy as enzyme concentrations are known to vary markedly from one animal species to another and even from one individual to another within species (WILLIAMS 1956).

An analysis of an organism content of an intermediate (A_4) does therefore not by itself give any information about the amount of the nutrient (A_1) that is required to maintain constant level of the intermediate. It follows that if two amino acids, let us call them A and B_1, in an organism or a tissue were going through reaction series of the type shown in equation (1), the ratio $\frac{A_1}{B_1}$ would not be predictable from the ratio $\frac{A_{se}}{B_{se}}$ obtained on analysis.

There is reason to assume that in most instances the substrate concentrations occurring within cells are small compared to the saturation values (KREBS 1957, DIXON and WEBB 1958, RINGLER 1959). The term $(V - V_1)$ A_1 in the denominator of equation (13) may therefore be negligeable compared to $V_1 \; K$. (This would be true also if V and V had nearly the same numerical value, a situation which seems, however, more unlikely.) Under such conditions equation (13) would read

$$(14) \qquad A_{se} = \frac{V \; K}{V \; K} \; A_1$$

Similarly the relation between B_{se} and B_1 could be written

$$(15) \qquad B_{se} = \frac{V' \; K'_2}{V' \; K'} \; B_1$$

and

$$(16) \qquad \frac{A_{se}}{B_{se}} = \frac{V_1 \; K_2}{V \; K} \; \frac{V'_2 \; K}{V' \; K'} \; \frac{A}{B_1}$$

According to the last equation, the ratio of the concentrations of two intermediates is proportional to the ratio of the concentrations of the substances from which they are formed. But the two ratios are not identical unless

$$(17) \qquad \frac{V \; K_2 \; V' \; K'}{V \; K \; V \; K} = 1$$

Suppose that on analysis the ratio $\frac{A_{se}}{B_{se}}$ was found to be 2. If the fraction of constants were $\frac{1}{2}$, the ratio $\frac{A_1}{B_1}$ would be 4.

It should be strongly emphasized that the discussion has been based on a model system (equation (1)) which is far too simple to describe adequately the metabolism of amino acids in the body. We have ignored, for instance, that the amino acids must be transported in one way or another through cell membranes before taking part in the metabolism. The mechanism for this process is not clear but it probably involves the interaction of the acid with "carriers". In the cell it is more probable that the amino acid will participate in a number of reversible bimolecular reactions rather than in an irreversible monomolecular reaction as stated in equation (1). An example of a reversible bimolecular reaction in which amino acids take part is their activation with adenosine triphosphate, a process that may be a prerequisite for protein synthesis (*cf.* CHANTRENNE 1958). It is not known whether this and other reactions leading to the incorporation of amino acids into proteins are "ordered" or "random" bimolecular reactions. In cases where the latter is true, the reaction rates will depend both on the first and the second power of the substrate concentrations (REINER 1959), thus making the ratio $\frac{A_{t_e}}{B_{t_e}}$ dependent on A and B in a complex fashion. Bimolecular reactions would in any case introduce more variables on which this ratio would depend. Even for amino acids like threonine or lysine that normally seem to serve no other purpose than as building blocks of proteins, a number of further complications can be conceived. The coupling of protein anabolism (and catabolism) to energy yielding processes, the influence of transit times and the size and composition of the metabolic pool of nitrogen, the fact that the enzymes participating in protein synthesis are themselves proteins, the effect of structural relationships in the "derivation" of rate equations, the dependence of protein synthesis on nucleic acid "synthesis", and other phenomena have all been left out of consideration here. The lack of knowledge of how amino acids are put together to form proteins makes it impossible at present to account for these factors. Therefore, the kinetic speculations outlined above merely serve to emphasize the hazard of drawing conclusions from only the concentration of an intermediate in a reaction sequence.

It is of interest in this connection to mention the kinetic aspects of protein synthesis that have been put forward by STEINBERG, VAUGHAN and ANFINSEN (1956) to account for the nonuniform protein labelling observed by them in experiments with radioactive amino acids. Assuming a stepwise synthesis of a protein via intermediate conjugates of the amino acids in the metabolic pool, they show that the radioactivity of an amino acid in the protein (A_2) depends on the radioactivity of the same acid in the pool (A_1) in the following manner

$$A_2 = k_1 A_1 \left(t + \frac{1}{k_{pe}} \left(e^{-k_{pe} t} - 1\right)\right) \tag{18}$$

In this formula k_1 and k_{pe} are rate constants and t is the time. If t is sufficiently large A_2 would be approximately equal to $k_1 A_1 t$. The same relation would hold for another

amino acid B and the ratio $\frac{A_1}{B}$ could be written

(19) $$\frac{A_2}{B_2} = \frac{k_{1a}}{k_{1b}} \cdot \frac{A_1}{B_1}$$

an expression resembling (16)

Discussion

Each amino acid takes part in the formation not only of one but a large number of proteins in the organism. It is known that all proteins in the body are not metabolized at the same rate (*cf* SCHOENHEIMER 1949 FRIEDBERG TARVER and GREENBERG 1948) It is by no means certain, that the proteins having the fastest turnover rate and therefore those which we daily have to replace and repair have the same amino acid composition as the entire animal or as muscle proteins as a whole. Even if the principle of the carcass analysis method were correct, it would thus be dangerous to use the analytical values of the amino acid composition of the whole animal or the whole muscle for the estimation of amino acid requirements.

When amino acids are set free in the body due to protein catabolism they mix with the amino acids of the metabolic pools which contain amino acids also of dietary origin. The amino acids of these pools are either used for protein synthesis or excreted unaltered or in a modified form. A slow breakdown and/or excretion of an amino acid derived from the catabolism of a tissue protein would mean that the amino acid could be reused for protein synthesis to a larger extent than an amino acid that is rapidly disposed of. Attention should be drawn to the fact that the renal clearance of amino acids varies markedly from one amino acid to another and in a fashion that bears no obvious resemblance to the amino acid composition of the entire animal (WRIGHT 1948, FOWLER *et al.* 1957 WILLIAMS 1959)

Some essential amino acids, *e g* methionine, tryptophan, phenylalanine and arginine (essential for rats, dogs and chicks) have other functions in the organism besides forming proteins. Methionine, for instance, participates in the formation of creatine, adrenaline, choline, N methylnicotinamide, spermidine and other compounds, and tryptophan can give rise to niacin and 5-hydroxytryptamine (*cf* FRUTON and SIMMONDS 1958) It is usual to express amino acid requirements by stating how many times they exceed the requirement of tryptophan. This is, of course, not a satisfactory method, since some of the tryptophan in the diet is used for purposes other than protein synthesis, and this to an extent that depends on the level in the diet of nutritional factors other than amino acids, *e. g.* niacin and vitamin B_6. Some animal species also convert tryptophan to niacin much more efficiently than others.

It should be strongly emphasized that the discussion has been based on a model system (equation (1)) which is far too simple to describe adequately the metabolism of amino acids in the body. We have ignored, for instance, that the amino acids must be transported in one way or another through cell membranes before taking part in the metabolism. The mechanism for this process is not clear, but it probably involves the interaction of the acid with "carriers". In the cell it is more probable that the amino acid will participate in a number of reversible bimolecular reactions rather than in an irreversible monomolecular reaction as stated in equation (1). An example of a reversible bimolecular reaction in which amino acids take part is their activation with adenosine triphosphate, a process that may be a prerequisite for protein synthesis (cf. CHANTRENNE 1958). It is not known whether this and other reactions leading to the incorporation of amino acids into proteins are "ordered" or "random" bimolecular reactions. In cases where the latter is true, the reaction rates will depend both on the first and the second power of the substrate concentrations (REINER 1959), thus making the ratio $\frac{A_{bt}}{B_{bt}}$ dependent on A and B_1 in a complex fashion. Bimolecular reactions would in any case introduce more variables on which this ratio would depend. Even for amino acids like threonine or lysine that normally seem to serve no other purpose than as building blocks of proteins, a number of further complications can be conceived. The coupling of protein anabolism (and catabolism) to energy yielding processes, the influence of transit times and the size and composition of the metabolic pool of nitrogen, the fact that the enzymes participating in protein synthesis are themselves proteins, the effect of structural relationships in the derivation of rate equations, the dependence of protein synthesis on nucleic acid synthesis, and other phenomena have all been left out of consideration here. The lack of knowledge of how amino acids are put together to form proteins makes it impossible at present to account for these factors. Therefore, the kinetic speculations outlined above merely serve to emphasize the hazard of drawing conclusions from only the concentration of an intermediate in a reaction sequence.

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$$A_p = k_{ps} A_s \left(t - \frac{1}{k_{ps}}(1 - e^{-k_{ps} t})\right) \qquad (18)$$

In this formula k_{ps} and k_{pa} are rate constants and t is the time. If t is sufficiently large A_p would be approximately equal to $k_{ps} A_s t$. The same relation would hold for another

amino acid B and the ratio $\frac{A_4}{B_3}$ could be written

(19) $$\frac{A_4}{B} = \frac{k_{14}}{k_{34}} \frac{A_1}{B}$$

an expression resembling (16)

Discussion

Each amino acid takes part in the formation not only of one but a large number of proteins in the organism. It is known that all proteins in the body are not metabolized at the same rate (*cf* SCHOENHEIMER 1949 FRIEDBERG TARVER and GREENBERG 1948) It is by no means certain, that the proteins having the fastest turnover rate and therefore those which we daily have to replace and repair have the same amino acid composition as the entire animal or as muscle proteins as a whole. Even if the principle of the carcass analysis method were correct, it would thus be dangerous to use the analytical values of the amino acid composition of the whole animal or the whole muscle for the estimation of amino acid requirements.

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$$A_t = k_1 A\left(t + \frac{1}{k_{pn}}\left(e^{-k_{pn}t} - 1\right)\right) \tag{18}$$

In this formula k_1 and k_{pn} are rate constants and t is the time. If t is sufficiently large A_t would be approximately equal to $k_1 \cdot A_1 \cdot t$. The same relation would hold for another

1952) How such large differences can occur is suggested by the influence of enzyme concentrations on the relation between A and A in formula (12) and the fact that activities of enzymes are — as has already been mentioned — known to vary markedly from one individual to another (WILLIAMS 1956) Enzyme activities also depend on the nutritional status of a given individual (WATERLOW 1959)

The amino acid composition of higher animals does not appear to vary with age (WILLIAMS *et al.* 1954) Although critical evidence for this seems to be lacking in the human, it appears reasonable to assume that it holds true also for this species. It should follow — according to the carcass analysis method — that the ratios of the amino acid requirements for growing children should be identical with the ratios of the concentrations of the amino acids in the adult. This argument has been upheld in particular by ALBANESE (1955 1959) who has provided evidence that the lysine tryptophan (L T) ratio in the diet of infants is optimal at approximately 6.3 1 which is the L T ratio of muscle proteins. The daily lysine requirement of infants is estimated by him to be 170—200 mg/kg body weight and the tryptophan requirement 30 mg/kg HOLT and SNYDERMAN (1956) seem to accept the same figure for tryptophan but their experiments with infants indicate a daily average lysine requirement of approximately 90 mg/kg This gives the same average L T ratio of 3 1 for the growth of infants as for the nitrogen balance in adults (*cf* ROSE 1957 LEVERTON 1959) a ratio that is, however only half that predicted by the carcass analysis procedure. Both ALBANESE (1959) and HOLT and SNYDERMAN (1956) have referred to body composition data while scrutinizing each others findings. It seems doubtful if this is a reliable basis for discussion

Various authors have repeatedly emphasized that the amino acid composition of different animals is practically the same, yet the average ratios of the amino acid requirements are not the same for the growth (or nitrogen balance) of the rat, chick, pig or human (*cf* WILLIAMS *et al.* 1954 ROSE 1957 HOLT and SNYDERMAN 1956, LEVERTON 1959) The rat, for instance, needs less leucine in relation to lysine than the human, although in the rat the ratio of leucine to lysine has actually been reported to be somewhat higher than in the human. Similar situations could be mentioned for other amino acids and other animal species. Also the attempts to calculate correlation coefficients between the amino acid content of various proteins in relation to muscle protein and the biological values of the same proteins (MITCHELL 1959 BOCCIO *et al.* 1952) have, in the author's opinion, given inconsistent results. One can, of course, argue that neither the amino acid analyses nor the estimations of requirements are as yet accurate enough to justify criticism of the carcass analysis method to be based on such failures, but it appears more likely that the reason for the discrepancies is of a fundamental nature. Further work — both theoretical and experimental — is needed to clarify the situation and to define the applicability of the method.

References

Addis, T L. J Poo and W Lew The quantities of protein lost by the various organs and tissues of the body during a fast. *J Biol. Chem.* 1936. *115.* 111—118.

Albanese, A. A. R. A. Higgons, G M. Hyde and L. Orto, Biochemical and nutritional effects of lysine-reinforced diets. *Amer J clin. Nutr* 1955. *3* 121—128.

Albanese, A. A., Protein and amino acid requirements of children, in Protein and amino acid nutrition. *Academic Press Inc.* New York 1959 419—475

Beach, E. F., B. Munks and A. Robinson The amino acid composition of animal tissue protein. *J Biol. Chem* 1943. *148.* 431—439.

Bocobo, D L., M. Shellenberg, C. R. Shaw and B. F Steele, Amino acid composition of some human tissues. *Arch. Biochem. Biophys.* 1952. *40* 448—452.

Chantrenne, H., Newer developments in relation to protein biosynthesis. *Ann. Rev. Biochem.* 1958. *27* 35—56.

Dixon, M and E. C. Webb, Enzymes. *Longmans, Green and Co.* London 1958.

Friedberg, F., H Tarver and D M. Greenberg The distribution pattern of sulfur-labeled methionine in the protein and the free amino acid fraction of tissues after intravenous administration. *J Biol Chem.* 1948. *173.* 355—361

Fowler, D I P M. Norton, M. W Cheung and E. L. Pratt Observations on the urinary amino acid excretion in man: the influence of ge and diet. *Arch. Biochem. Biophys.* 1957 *66.* 452—466.

Fruton J S and S. Simmonds, General Biochemistry *John Wiley & Sons Inc.* New York. 1958.

Holt Jr. L. E. and S. E. Snyderman, The amino acid requirements of children, in Some aspects of amino acid supplementation. *Rutgers University Press.* New Brunswick. 1956. 60—68.

Krebs, H. A. Control of metabolic processes. *Endeavour* 1957 *XVI* 125—132.

Leverton, R. M. Amino acid requirements of young adults, in Protein and amino acid nutrition. *Academic Press Inc* New York. 1959 477—506.

Mitchell, H H., Some species and age differences in amino acid requirements, in Protein and amino acid nutrition. *Academic Press Inc.* New York. 1959 11—43.

Munks, B., A. Robinson, E. F Beach and H H Williams, Amino acids in the production of chicken egg and muscle. *Poultry Sci.* 1945. *24* 459—464

Reiner, J M., Behaviour of Enzyme Systems. *Burgess Publ. Co.* Minnesota. 1959.

–, W C. The amino acid requirements of adult man. *Nutr Abstr Rev.* 1957 *27* 631—647

Schoenheimer, R., The dynamic state of body constituents. *Harvard University Press.* Cambridge Mass. 1949

Sharpenak, A. E., The requirements of man for certain amino acids. *Voprosy Pitania.* 1957 *16.* 9—17 Taken from *Chem. Abstr* 1958. *52.* 6526—6527

Steinberg, D M. Vaughan and C. B. Anfinsen, Kinetic aspects of assembly and degradation of proteins. *Science.* 1956. *124* 389—395.

Tappan, D V   U J Lewis, U D. Register and C. A. Elvehjem Niacin deficiency in the rhesus monkey *J Nutr* 1952. *46.* 75—85.

Waterlow J C., Protein nutrition and enzyme changes in man. *Fed. Proc.* 1959. *18.* 1143—1155.

Williams, H H., L. V Curtin, J Abraham J K. Loosli and L. A. Maynard, Estimation of growth requirements for amino acids by assay of the carcass. *J Biol. Chem.* 1954. *208.* 277—286.

Williams, R. J   Biochemical individuality *John Wiley & Sons Inc* New York. 1956.

Williams, R. J   Individuality of amino acid needs in protein and amino acid nutrition in Protein and amino acid utrition. *Academic Press Inc.* New York. 1959 45—56.

Wright L. D., Renal clearance of essential amino acids. *Trans. N Y Acad. Sci.* 1948. *10.* 271—277

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Further Studies on Pigment Migration and Sensitivity Changes in the Compound Eye of Nocturnal Insects

By

C. G BERNHARD and D OTTOSON

It has been shown that the dark adaptation in noctuid moths proceeds in two phases (BERNHARD and OTTOSON, 1959 1960 a) and comparative histological studies have brought evidence that the increase in sensitivity during the second phase is mediated by the outward movement of the retinal pigment (BERNHARD and OTTOSON 1959 1960 b)

Continued electrophysiological and histological studies on the relationship between the positional changes of the retinal pigment and the sensitivity of the eye have now been carried out on the isolated eye of the noctuid moth *Cerapteryx graminis*. In 21 eyes the sensitivity changes were measured electrophysiologically (see BERNHARD and OTTOSON 1960 a) during dark adaptation and during adaptation to light of different intensities after exposure of the eye to bright light (pre-adapting illumination 1—3 min relative intensity — 1) Fig 1 A shows the typical discontinuous dark adaptation curve, the second phase of which starts about 9 min after cessation of the bright light. Curve 1 B, showing the sensitivity changes during the exposure of the eye to an adapting light with a relative brightness of — 3 4 has no corresponding second phase. The lack of this phase is not due to impairment of the eye since there is a sudden fall in the curve as soon as the adapting dim light is shut off (at vertical arrow) Curves C and D obtained with adapting lights w th lower light intensities (— 4.6 respectively — 6.4) show a second phase which is more pronounced at the lowest intensity of the adapting light. As expected cessation of the dim light (vertical arrow) is followed by a further decrease in the threshold. All curves obtained with adapting light intensities above — 4 6 (between — 2.2 and 4.3) showed no second phase, whereas 14 curves out of 16 obtained with adapting intensities between — 4 6 and — 7 showed a second phase which in general was greater at lower intensities. Whereas the second phase ppears with a long latency during dark adaptation after bright pre-adapting light (curve A) the further fall in the curves appears without appreciable delay when darkness follows exposur to light of lower tensities (curves B, C, D)

Dark adapted eyes as well as eyes which had been adapted for 20 min to light of different intensities, ranging from — 3 4 to — 7 were fixed in Bouin-Duboscq-Brasil's solution and sta ned with haemalum-eosin. There was no

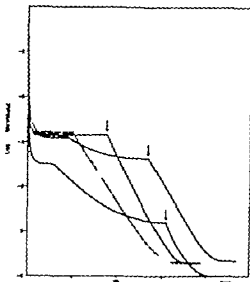


Fig. 1. Dark-adaptation curve of *Cossus* (A) and curves showing the relation between threshold intensities and time of adaptation to light of different intensities (B, C and D; for further description see text).

pigment retraction in the eyes which had been exposed to light intensities above — 4.6, whereas a retraction of varying degree was seen in the eyes which had been exposed to intensities between — 4.6 and — 6, maximal retraction being found in most eyes exposed to intensities below — 6.

The facts 1) that neither the second phase of the adaptation curve nor the pigment migration were obtained when the eyes were exposed to light with intensities above a certain value (— 4.6), whereas both the second phase and the pigment migration were found at light intensities below this value and 2) that there is a relation between the magnitude of fall of threshold during the second phase and the degree of pigment retraction with dim light of different intensities, give further evidence that the second phase of the adaptation curve, is correlated to the outward movement of the pigment. Taking this conclusion into consideration, the curves in Fig. 1 would show that the pigment retraction starts after a long latency of several minutes after exposure of the eye to bright light, whereas the retraction begins without appreciable delay when the eye has been exposed to lower light intensities.

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References

BERNHARD, C. G. and D. OTTOSON, On the mechanisms of dark adaptation in day- and night-insects. *Acta phys. scand.* 1959. *47*. 383—384.

BERNHARD, C. G. and D. OTTOSON, Comparative studies on dark adaptation in the compound eyes of nocturnal and diurnal Lepidoptera. *J. gen. Physiol.* 1960 a. *44*. 195—203.

BERNHARD, C. G. and D. OTTOSON, Studies on the relation between the pigment migration and the sensitivity changes during dark adaptation in diurnal and nocturnal Lepidoptera. *J. gen. Physiol.* 1960 b. *44*. 205—215.

ERRATA

p 19 line 4 from below 10^3 c. p. s. should be replaced by 10 c. p. s.

p 63 line 3 from below see footnote p. 000 should be replaced by see footnote p 62

p 77 Boeminghaus & Kochmann last column 0.15 should be replaced by 0.015 and 0.23 should be replaced by 0.023.

p 77 Bennett et al column 7 0.1–0.15 mM should be replaced by 1–1.5 mM.

p. 135 equation (1) exponent c should be replaced by s
line 14 from below c should be replaced by s

p 143 equation (8) should be replaced by $s = 7 \times 10^{-7}$ sec^{-1}
last line cm^{-3} × sec^{-1} should be replaced by sec^{-1}

p. 144 line 8 1.5 should be replaced by 2.1
equation (10) 7.5 should be replaced by 5.3

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From the Pathology Department, Tissue Dynamics Laboratory, University of Texas Medical Branch, Galveston, Texas, U.S.A.

The Persistence and Regularity of Electrical Activity in Whole Heart Explants

By

A. W. B. Cunningham, N. O. Lunell and B. J. Rylander

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Abstract

Cunningham, A. W. B., N. O. Lunell and B. J. Rylander. *The persistence and regularity of electrical activity in whole heart explants.* Acta physiol. scand. 1961. 52. 101—107. — The electrical persistence and regularity of 143 chick embryo whole heart explants during nine days in culture is described. Details are given of culturing and surveillance of explants and handling of data. Over 90% of explants showed continuous electrical activity for nearly all of the first three days in culture. This percentage fell 6 % by the end of day three, 6 % during day four, 17 % on day five and more slowly thereafter. These changes possibly reflected inadequacies in the supernatant. The frequency and magnitude of change in the number of active cultures from one 15 second survey to the next slowly increased with a maximum of 1.2 cultures. It seemed that the failure of hearts to produce potentials was usually temporary and occurred in a rhythmic fashion. The difference between consecutive half-hour averages of the number of cultures showing activity during 15 out of every 20 seconds was relatively high initially, dropped during the second day then rose again. It was always small compared to the total number of cultures involved. Comparisons between the persistence of electrical function in whole heart explants in various supernatants showed that the one used in this study was least inadequate.

The contractile activity of chick embryo hearts has been previously followed by microscopic observation and recording (v. Bodendorff 1951). These observations were made intermittently since no automatic recording technique was used. In the present investigation, observations were made on 143 whole

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chick embryo hearts for up to 216 hours after explantation using a more continuous automatic recording technique. The change in the number of hearts showing electrical activity as a result of the passage of time was measured.

Techniques

The 143 hearts were observed in 4 groups (the incubators held a maximum of 40 cultures) (A) 38 hearts observed from 124—213 hours after explantation, (B) 33 hearts observed from 4 / —230 hours after explantation, (C) 36 hearts observed from 3 /$_s$—145 hours after explantation and (D) 36 hearts observed from 4 /$_s$—150 hours after explantation.

Each heart was explanted from a 7-day embryo chick onto a 36 gauge, bare platinum electrode in the angle of an L-shaped piece of cellulose sponge held onto coverglass by Diatex (CUNNINGHAM and LUNELL 1960) Diatex is a toluol solution of modified crylic cid compound which, when properly dried, gives a hard transparent film adherent t glass and, in our experience, non-toxic to heart cultures. Each explant on its electrode and coverglass was placed in a separate culture chamber equipped with 36 gauge platinum reference electrode and a flow system giving a uniform flow of 1 ml per day (CUNNINGHAM and LUNELL 1960) The culture chamber and flow system were clipped onto a metal frame on which the electrodes were connected, by insulated connectors, to a shielded cable. The whole system was kept in an incubator at 37° C.

Supernatant — This was a 0.25 % solution of human serum protein in the following balanced salt solution (Tissue Dynamics Laboratory Balanced Salt Solution, TDLI)

	g/l		meq l
Potassium Chloride	0.28	Potassium	4.15
Sodium Chloride	7.0	Sodium	134.0
Calcium Chloride.	0.28	Calcium	5.04
Magnesium Sulphate	0.15	Magnesium	2 44
Disodium Phosphate	0.08	Phosphates	1 44
Monopotassium Phosphat	0.04		
Sodium Bicarbonate	1.05	Bicarbonat	12.5
Sodium Acetate	0.05	Sulphate	2 44
Dextrose	1 75	Chlorides	126.5
Water to one liter			

Collection of Data

All heart explants in the study were surveyed automatically using an lectronic instrument which detected and recorded the total number of hearts giving rise to minimum of one 0 05 mV potential during the first 15 out every 20 sec. Potentials arising in any of the hearts were amplified and fed into channel in the electronic recording apparatus (there was separat channel for each heart under surveillance) where the first impulse of more than 0 05 mV to arrive activated an electronic sw tch which then remained activated for the whole 15 sec survey At the end of the 15 sec survey a measurement was made of the total amount of current flowing through all the activated switches. This value was registered on stripchart recorder as a straight line (Fig. 1) and its length was directly proportional to the number of heart explants which had given rise to one or more potentials greater than 0 05 mV during the past 15 sec. Following the 15 sec survey there was a 5 sec silence to allow the detecting mechanism and recording pen to reset.

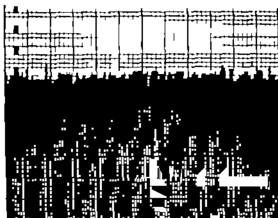


Fig. 1 A half hour strip of the read-out from the automatic electronic monitor. Each peak represents the number of explants which have given rise to one or more potentials of greater than 0.05 mV during the previous 15 seconds. Each horizontal line represents one explant.

Processing of Data

Using the above apparatus, over two million observations were made on 143 cultures in over one hundred thousand 15 sec surveys. A sequence of values representing the averages of all 15 sec surveys for each half-hour were obtained by planimetry. This was done by measurement of the area between the baseline and a line joining the peaks of the lines representing the values obtained from each 15 sec survey during that half-hour. To facilitate comparison of the results from different groups of cultures, the planimetric average for each half hour was expressed as a percentage of the planimetric average for a half hour during which it was judged that every culture in that particular group showed electrical activity every 15 sec.



Fig. 2. Y axis — Half hour averages of the number of cultures showing electrical activity during 15 out of every 20 seconds (consecutively), expressed as a percentage of the total number of explants showing activity at the beginning of the experiment. From the time of explantation till 143 hours Groups B, C and D were involved, from 143—215 hours Groups A and B were involved and from 215—240 hours only Group B was involved. Dotted line-actual half hour averages, Solid line — 5 hour running average. X axis — Time in hours.

The processing of the data from Group A provided a special difficulty since the planimetric values for a half-hours full activity in all cultures in this group (which would have been the 100 % value) was not available. Therefore, in order to assign a percentage value to each half-hour planimetric verage in this group, the mean of all half hour planimetric values for Group A from 144—168 hours after explantation was arbitrarily given, as a percentage value, the mean of all the percentage persistence values for the period 144—168 hours in culture from Group B. Thus a relationship between half-hour averages of the 15 sec surveys and percentage electrical persistence was established and from it percentage values could be worked out for each planimetric value for Group A. Also, since the percentage values for Group B between 144—168 hours in culture were higher than those likely to have been found in Group C or D the percentage values for Group A were also higher Hence the curves representing the survival of electrical potential production ran at a higher level for the period after 150 hours in culture (when t is constructed from data from Groups A and B) than before 150 hours in culture (when it is constructed from data from Groups B, C and D) (Fig 2)

Results

Data on Persistence of Electrical Potential Production

The curve constructed from the average of the half hour planimetric values for the four groups of cultures (B C, and D before 150 hours and A and B after 150 hours) is given in Fig 2 The heavy line which represents the 5 hour running average, shows that there was an increase of 3 % in the number of functioning cultures during the first 24 hours in culture. During almost all of the next 45 hours, the average percentage of active cultures remained over the 90 % level but had a drop of 4 %. The period from 69—92 hours in culture showed a slightly longer drop in the persistence of electrical activity (6 %) while the sharpest fall of all was seen between 92 and 117 hours in culture (20 %) From 118 till 144 hours the curve shows a flattening out with only a 4 / % drop The principal reason for the rise in level between 145 and 153 hours has already been discussed. However the figures for Group B alone, show that there is indeed a slight rise in the proportion of active cultures at this time amounting to about 4/5ths of that seen in Fig 2 for this time in culture (i.e 8 % instead of 10 %) From this time till 176 hours in culture there is a drop of 10 / in the number of active cultures and from 176 till the end of the curve a drop of 5 °

Data on Regularity of Electrical Behavior

The data available in this study also provides information on the regularity of the contractile behavior of whole hearts in culture Fig 1 is a half hour strip of the straight line read-outs, each of which represents the total number of hearts producing a potential of more than 0 05 mV during a 15 sec survey period. This strip was taken at random from those for this study and shows the remarkably uniform behavior of the hearts in this respect. An examination of a similar strip of recording taken for the period 8.30—9 00 p.m during each day for

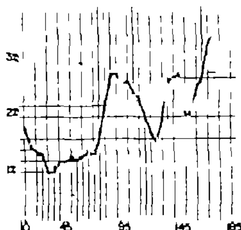


Fig. 3. Y axis — The five hour running average of the change (increase or decrease) in the number of explants showing electrical activity during 15 second surveillance. This running average was constructed from the combined half hour averages from all four groups and corrected to represent percentage of the total number of hearts actually showing activity at the time of the change.
X axis — Time in hours.

each of the four groups of cultures shows that the number of cultures showing electrical activity per fifteen second survey varies to a relatively great extent during the first 24 hours. During this time there is an average change of 0 45 in the number of active cultures, between successive 15 sec surveys during the half hour During the next 48 hours this value dropped to 0.3 cultures then rose again till by 144 hours it was about 0.5 cultures. However for every increase in the number of electrically active cultures, there was a subsequent and almost equal decrease so that the ultimate net change in the number of cultures was not large.

Fig 3 shows the 5 hour running average of the change (increase and decrease) in the number of electrically active cultures.

The running average was constructed from the half-hour averages for all four groups of cultures. It is corrected to represent a proportion of the total number of hearts actually showing electrical activity at the time of the measurement of the change.

Discussion

Our present knowledge of the factors governing the survival of electrical potential production in heart explants is small and we have been unable to find truly comparable published data on this problem, save in papers previously published from this laboratory Hence discussion is necessarily limited and the paper is largely descriptive.

The form of the curves for the individual groups of cultures had a definite family resemblance to that of the overall average behavior The form of the curve in Fig 2 is probably the result of a combination of different causes of myocardial damage such as

a) the effects of the products of mechanical tissue damage inflicted during explantation,

b) malnutrition from poor physical distribution of food and oxygen,
c) lack of essential materials originally in short supply
d) diffusion of vital metabolic intermediaries through damaged cell membranes.

In this curve, the initial 3 % rise during the first 24 hours was probably due to recovery from the trauma of culturing. The 45 hour plateau above 90 % electrical persistence which follows suggests that, during this time, there was a sufficient supply of vital metabolic materials. Then the sharp drop which progressively becomes less, presumably reflects inadequacy in the artifical environment. While the nutritional demand from a relatively large number of active cultures was high early in this phase, there was a sharp decline in the ability of some cultures to maintain electrical persistence. Later in this phase there were fewer active cultures and presumably lesser nutritional demand hence the decline became less.

Some further comments on this curve will be found in other parts of this discussion.

The Regularity of Electrical Activity in Cultures

The method used to record activity in this study was such that a change in the number of active cultures may mean that a culture was

a) starting for the first time,
b) stopping finally
c) beating irregularly so that it was constantly making reentries,
d) beating regularly less than once per 15 sec so that it did not appear in every 15 sec scan.

The number of electrically active cultures per 15 sec survey shows a trend of increased variation after 72 hours even when a daily sample of only one half hours activity is examined. The level of variation is small when compared with the total number of cultures involved. Also, the total number of cultures showing electrical activity per survey period increases nearly as often as it decreases. This suggests that a culture usually lost electrical activity only for a short time. The progressive increase in the amount of variation in the number of active cultures as they aged is seen in Fig. 3. A comparison of Fig. 2 and 3 shows that the greatest change in the number of active cultures co-incided with the greatest fall in the total number of active cultures. Thus, in general there is a progressive increase in the frequency and magnitude of irregularity in the number of electrically active cultures as they aged.

The Effect of Electrolytes on Electrical Persistence

The effects of the electrolyte composition of the supernatant on the persistence of electrical activity in the cultures can be measured by a comparison between the data from this study and that from previous similar studies in this laboratory. A previous paper (Estborn et al. 1958) gave information on the

electrical persistence in whole heart explants cultured in synthetic supernatant 199 The criteria for a 192 hour survival (the production of a 0.02 mv potential during one of 29 7½ second surveys between 192 and 216 hours in culture) would probably be regarded as a 216 hour survival in the present study Using synthetic supernatant 199 only 61 out of 258 hearts (24) showed adequate electrical function for 215 hours in culture. In the present study more than 50 % cultures showed electrical activity at 216 hours in culture. Thus, our present supernatant permitted twice as high an electrical persistence in whole hearts in culture than did synthetic supernatant 199

Another previous study (CUNNINGHAM, KLEUTCH and HERBST 1960) gave a comparison of the persistence of electrical activity in supernatants of differing potassium levels. It concluded that, of those tried, the supernatants with a potassium level of 4.0 meq/l gave the best result. Our present supernatant has a potassium level of 4 15 meq/l and its levels of sodium, magnesium, calcium and phosphate are more physiological. The proportion of cultures showing electrical persistence at 168 hours in the 4.0 meq/l was 52 % In the present study t is between 62 and 73 Thus the regulation of all of the electrolytes to physiological levels causes a 20 % greater electrical persistence at 168 hours in culture.

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References

VON BONSDORFF R., The electrogram of embryonic heart muscle cultivated in itro and its relation to the electrocardiogram of the embryonic heart. *Acta physiol scand.* 1951 22. Supplementum 75.

CUNNINGHAM, A. W B. and W HERBST A simplified quantitative tissue culture flow system. *Lab Invest.* 1960. 9. 384—387

CUNNINGHAM, A. W B., K. KLEUTCH and W HERBST Effect of supernatant potassium level on cardiac activity in quantitative tissue culture. *Nature* (Lond.) 1960. 186.

CUNNINGHAM, A. W B. and N O LOVELL, Further simplification of supernatant flow system for tissue culture. Experientia (Basel) 1961 *17* 94.

EBBORN, B. D A. W B. CUNNINGHAM, L. NEILL, G. THOMAS, H. N THOMAS, P COCHRANE and S. G. STEPHENS. Quantitative tissue culture technic as applied to the study of cardiac potentials. *Lab. Invest.* 1958. *7* 524—532.

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The Discharge in Single Touch Receptors Elicited by Defined Mechanical Stimuli

By

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Abstract

Höglund G and U Lindblom. *The discharge in single touch receptors elicited by defined mechanical stimuli.* Acta physiol scand. 1961 52 108—119 — To understand how a touch stimulus is coded in nerve impulses it is fundamental to know the significance of its wave form. This has been studied in the toad by means of a special technique for stimulation of individual touch receptors in situ with simultaneous recording of the skin displacement. Stimuli of various gradients, amplitudes and durations were applied to the skin surface over the receptor and the impulse discharge in the afferent fibre was recorded from thin filaments of the dorsal root.

The threshold amplitude was remarkably independent of the stimulus gradient. When the latter was increased gradually from the minimum effective value (critical slope) and upwards, the threshold fell sharply at first but then remained at the same level (Fig. 3 and 4)

Prolongation of a threshold stimulus by adding a plateau phase to the initial moving phase did not result in a repetition of the response and did not make it possible to reduce the amplitude (Fig. 1) The repetitive response usually obtained on suprathreshold stimulation was either limited to the moving phase or had the highest impulse frequency during this phase (Fig. 2)

Since the strongest excitation occurred during the moving phase of the stimuli, it was concluded that the deformation is the adequate stimulus. The deformed state is normally followed by no response at all or in some receptors, by a rapidly adapting discharge.

In a recent paper the excitability of touch receptors of the type that is connected to coarse afferent fibres was studied in situ on the toad (Lindblom 1958) These receptors were shown to have a relatively refractory period of a con-

siderable duration, and the refractoriness left behind by the individual impulses was found to summate during repetitive firing so as to counteract further discharge from the receptor. It was considered to be of interest to investigate quantitatively in the same type of preparation other factors that influence the impulse frequency and the duration of the discharge. Some of these factors are the excitability properties of the receptors while others belong to the stimulus. The present paper describes the effect on the discharge of varying the form of a defined mechanical stimulus. A preliminary report has been given at the X Scandinavian Congress in Physiology in Oslo (Höglund and Lindblom 1960).

There are several earlier observations that concern the relation between the characteristics of the stimulus and the discharge in touch receptors of the type supplied by coarse afferent fibres. As was originally shown by Adrian and Zotterman (1926) in the cat, the typical response in the coarse afferent fibres from the skin on application of slight pressure is a short-lasting high-frequency burst of impulses. Adrian, Cattell and Hoagland (1931) found that the discharge of the touch receptors, studied in nerve-skin preparations from the frog, could be reduced to a few impulses if the stimulus, which consisted of an air current, was applied suddenly. They assumed that impulses are set up only during the actual period of movement of the skin. The same authors also pointed out that a certain abruptness is essential to stimulation, and later Gray and Malcolm (1951) made direct measurements of the critical slope for discharge in the same type of preparation. For stimulation they used a piezo-electric crystal which was placed on the inside of the skin so as to stimulate the termination of a single axon.

The advantages with the technique we have used, apart from the receptors being studied in situ with intact circulation, are that the adequate stimulus can be localized to a single touch-sensitive point in the receptive field and defined in terms of displacement of the skin surface. The type of preparation used has not allowed a direct study of local potentials in the receptor region or the accommodative properties of the fibre endings. This is a clear limitation from a theoretical point of view. For the present purpose, however, the essential thing is that the message in the afferent fibres could be related quantitatively to the physical characteristics of the stimulus. In order to obtain skin displacements of variable slope, amplitude and duration a specially designed mechanical stimulator has been used (Haapanen 1960).

Methods

All experiments were performed on decerebrate toads (*Bufo bufo*). 101 receptors belonging to 65 tactile units (see Lindblom 1958) with their receptive fields on the lower leg or plantar surface of the foot were studied. Action potentials were led off from single afferent fibres in the dorsal root. The stimulator (Haapanen 1960) gave linearly rising mechanical pulses, the rate of rise of which was continuously variable between 0.8 and 200 mm/sec. The movement could be interrupted at any moment during the rising

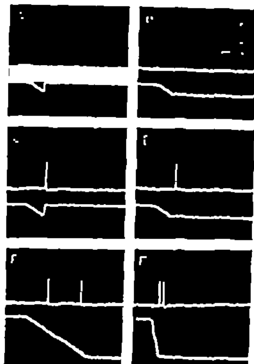


Fig. 1. Records from dorsal root fibre (upper beam) and capacitance meter (lower beam) illustrating the ineffectiveness of steady stimulation at subthreshold (A and B) threshold (D) and suprathreshold (E and F) stimulus amplitude. F shows also the effect of increasing the stimulus gradient. Horizontal bar 50 msec. Vertical bar 100 μ. Stimulus gradient in A—E 1.8 mm/sec, in F 13 mm/sec. From touch receptor on proximal part of plantar surface.

phase and the stimulator brought back to zero position or maintained at the level of interruption for a variable length of time so as to give a steady displacement of the skin. The maximal amplitude of movement was 200 μ. The stimuli were applied to the skin surface by means of a small blunt rod, which was adjusted so as to produce a vertical displacement of the skin overlying the receptor. Before stimulation, the stimulator which was mounted on a micromanipulator was put in contact with the skin without compressing it. Care was also taken not to stretch the skin when the leg was fixed prior to stimulation (cf. LOEWENSTEIN 1956). The displacement of the skin surface was recorded on each stimulation by means of a capacitance meter as has been described previously (LINDBLOM 1958, p. 12).

Results

On stimulation with *threshold amplitude* the response always consisted of a *single impulse. Fig. 1 C shows the response to a linearly increasing* mechanical stimulus of threshold amplitude. Maintained displacement, as in Fig. 1 D did not result in a repetitive discharge and was not accompanied by a lowering of the threshold amplitude[1]. This result shows that the moving phase is the effective part of the stimulus and that the adequate stimulus for these receptors

[1] In Fig. 1 C and D, as well in subsequent records, the impulse appears in the record *after* the end of the moving phase of the stimulus. This delay corresponds to the time for conduction of the impulse from the receptor to the site of recording in the dorsal root (cf. LINDBLOM 1958).

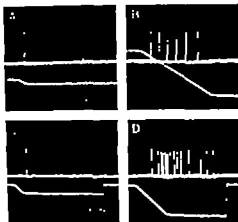


Fig 2. Typical discharges following threshold (A and C) and suprathreshold (B and D) stimulation of two different touch receptors which both are less rapidly adapting than the receptor illustrated in Fig 1 A and B from receptor on lateral pad of plantar surface. Horizontal bar 100 msec. Vertical bars 100 μ. C and D also from receptor on lateral pad but from another experiment. Horizontal bar 100 msec. Amplitude scale same as in B. Stimulus gradient in all records 0.8 mm/sec.

is th deformation. This is further emphasized by the results of sub- and supra threshold stimulation. In Fig 1 A the amplitude of the stimulus is slightly less than the threshold value in C. Record B shows that no response was obtained when the displacement was maintained at the same level as in A. Thus, steady stimulation could not yield the small degree of excitation needed for discharge when t had been brought to a near threshold level by the preceding moving part of the stimulus.

On *suprathreshold* stimulation the most rapidly adapting receptors responded with only a few impulses. For the receptor illustrated in Fig 1 *e.g.*, an increase of the amplitude of the stimulus to 4 times the threshold value did not result in more than two impulses (record E) From other receptors a burst of several impulses could be obtained. The discharge from such a receptor is illustrated in Fig 2 A and B, which show the response to threshold and supra threshold stimulation, respectively Even in this case the discharge is limited to the moving phase of the stimulus. Fig 2 C and D show the response from a third type of receptor which discharged also during the plateau phase of the stimulus on suprathreshold stimulation. As seen in D the impulse frequency is highest in that part of the discharge which has been initiated during the moving phase. This type of less rapidly adapting touch receptor was the only one that responded substantially to steady stimulation. Even so, however the discharge usually came to an end in less than a second except for single randomly discharged impulses.

The discharge in the less rapidly adapting touch receptors of the type illustrated in Fig 2 C and D was often potentiated if the skin was displaced prior to stimulation by lowering the stimulator 100—300 μ from the ordinary zero position where the stimulator rod was just in contact with the skin surface. Under these circumstances there were two receptors whose discharge lasted a little more than half a minute on steady stimulation. For the rest of the receptors

Table I Critical slope of 8 receptors

mm/sec.	rheob./sec.	mm/sec.	rheob./sec.
0.8–1.6		2.5– 2.6	
0.8–2.1		2.5– 3.4	
0.8–1.6		2.5– 4.2	
0.8–2.6	3.3–11	2.6	38
		2.6– 3.1	
1.0	22		
1.3–2.1		2.8	
1.4–1.6		2.8–3.0	
1.4–2.1	17–26	2.9–34.0	97–1 100(?)
1.4–3.2		3.8– 5.0	
1.9	42	3.9	
2.1–2.8	31–41	10.0–11.0	160–180
2.1–3.5	17–29	10.0–12.0	180–200
2.3–2.6		10.0–15.0	150–200
2.4–4.8		11.0–40	32–190

the potentiation consisted of a slight or moderate increase of the frequency and duration of the discharge. The sensitivity of the most rapidly adapting receptors was either uninfluenced or lowered by a preset displacement. None of the receptors was thus found to be slowly adapting in the sense that a long-lasting or stationary discharge was obtained with stimulation and recording conditions that may be considered as physiological. The material seems to be large enough to allow the conclusion that more or less rapid adaptation characterizes all low threshold mechanoreceptors in the toad's skin supplied by coarse afferent fibres.

Some receptors showed an off response consisting of a single impulse which appeared when steady stimuli with a duration of a few hundred msec. had been applied and were sharply cut off. The occurrence of an off-response was apparently not related to the rate of adaptation or the threshold of the receptor. An after-discharge, which characterize touch receptors connected to small fibres in the cat (ZOTTERMAN 1939), or a spontaneous discharge in the absence of mechanical stimulation was never observed.

The observations in the present investigation all show that the strongest excitation occurs during the period of deformation and that many, perhaps most, of the toad's touch receptors supplied by coarse afferent fibres do not normally discharge at all during steady stimulation irrespective of the stimulus strength.

If the stimuli to the receptor were to be effective, the rate of displacement had to be at or above a certain critical value. A lowering of the rate below this value could not be compensated for by an increase of the amplitude of the displacement. 58 of the 86 receptors which were tested for the critical slope (c.s.) were found to discharge on stimulation with the lowest rate of displacement

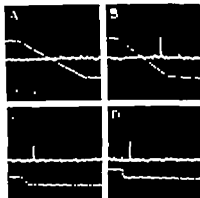


Fig. 3. Records illustrating the effect on the threshold of varying the stimulus gradient. This is in A 1.7 mm/sec., below the critical slope, in B 2.2 mm/sec, same as the critical slope, and in C and D, 4.1 and 11 mm/sec, resp. From touch receptor on proximal margin of plantar surface.

which could be obtained from the stimulator It can therefore only be stated that the *c.s.* of these receptors was at or below 0 8 mm/sec. For the remaining 28 receptors the *c.s.* was between 0.8 and 40 mm/sec.

The *c.s* very often showed temporal fluctuations. For some receptors identical values were obtained when the test was repeated several times, but the typical finding was that the *c.s* varied continuously back and forth within certain limits when the same receptor was followed for some length of time. Significant variations were often found from one minute to another As a consequence of the temporal fluctuations, the *c.s.* of a particular receptor should be given as a range rather than a single value. In table 1 the extreme values for all 28 receptors has been listed to give an idea of the magnitude of the fluctuations as well as the absolute values for the *c.s* Some of these are also given in rheobases/sec. The threshold on stimulation with a steep gradient (corresponding to the horizontal plateau of the curve in Fig 4) was taken as rheobasic strength. The observation time was about half an hour for each receptor

The temporal fluctuations could not be correlated with any special circumstance in the experimental situation, such as temperature or moisture of the skin, and therefore they most probably indicate a spontaneous fluctuation in the excitability of the receptor endings. Another possibility is that they reflect centrifugal regulating activity of sympathetic fibres to the skin (LOEWENSTEIN 1956 b)

In one series of experiments the threshold amplitude was determined at various rates of displacement above the *c.s.* and the typical result is illustrated in Fig 3 and 4 Fig 3 A *shows stimulation at the rate of 1 7 mm/sec. This* stimulus was ineffective in spite of having the maximal amplitude that could be obtained from the stimulator (260 μ) When the rate of the displacement was successively increased, a propagated response was suddenly obtained at the rate of 2.2 mm/sec (Fig 3 B) As seen, the impulse appears in the dorsal root fibre during the downward movement of the stimulator This means that the stimulus amplitude was suprathreshold.

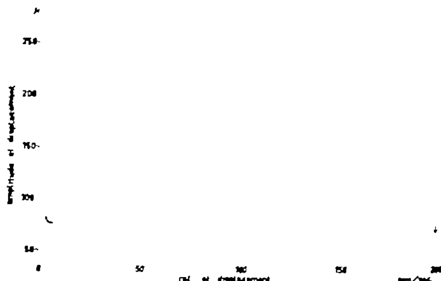


Fig. 4. Curve showing the typical relation between threshold amplitude and gradient of stimulus. From touch receptor on plantar surface. Critical lope 2.3 mm/sec.

By reducing the latency by the calculated time for conduction of the impulse from the receptor level to the site of recording n the dorsal root (cf. LINDBLOM 1958) it could be determined that the impulse in Fig 3 B was discharged from the receptor approximately 85 msec after the beginning of the displacement of the skin surface. This means that the threshold amplitude amounted to 195 μ on stimulation at the *c.s*

The threshold was then determined at various higher rates. This was done by reducing the amplitude of the stimulus until the response disappeared. (This method was easier to use than latency measurements but equally reliable since as described above the impulse is initiated during the phase of downward movement and not during the plateau phase of the stimulus) In Fig 3 C where the rate of displacement had been increased to 4 1 mm/sec, the threshold amplitude was 55 μ, *i e* considerably lower than at the *c.s* A further increase in the rate of displacement, however did not result in a further reduction of the threshold (Fig 3 D) The relation between the gradient and the threshold amplitude of the stimulus is shown in detail in the curve n Fig 4 which is from another receptor As seen the threshold falls very rapidly at rates just above the *c.s* and thereafter remains at the same level throughout the whole range of higher rates.

The conclusion to be drawn from these experiments is that, as soon as the gradient of the stimulus is well above the *.s.*, the receptor is insensitive to a further rise of the gradient.

On *suprathreshold* stimulation the gradient of the stimulus is very relevant for the various features of the discharge W th stimulus rates at or near the critical

slope the latency is usually relatively long In Fig 3 B, *e.g.*, the receptor delay was about 85 msec. Another consequence of a low stimulus rate is that the discharge is limited by the adaptation which comes in already during the phase of deformation. This can be seen even for the receptors that are prone to fire repetitively An increase of the gradient is followed by a shortening of the latency and an increase of the discharge frequency (Fig 1 E and F) The extent of the frequency increase will primarily be determined by the balance between excitation and refractoriness. The latter which summates on repetitive firing, will be more relevant than adaptation for the limitation of the discharge on stimulation with steep gradients.

Discussion

The suggestion made by ADRIAN CATTELL and HOAGLAND (1931) that impulses in coarse tactile fibres are set up only during the period of movement of the skin has been largely confirmed in the present experiments. These have shown that the strongest excitation occurs simultaneously with the deformation of the endings while the deformed state is accompanied by a relatively weak discharge or not discharge at all. It may be pointed out that, although the technique we have used allows a quantitative evaluation of the stimulus in terms of displacement of the skin surface, the type or extent of the deformation of the mechano-sensitive structures within the skin cannot be defined. The whole tissue in which the endings are embedded is displaced and deformed by the stimulus and the deformation of them may include both compression bending and stretch (cf LINDBLOM 1958 Fig 3)

During steady stimulation of touch receptors connected to coarse afferent fibres, the rapid adaptation is the most conspicuous and, from a physiological point of view obviously the most relevant event. If the propagated response is taken as the only index of excitation this would be zero in the adapted state in which no impulses are elicited. Subliminal changes of the excitability may however exist in the absence of any propagated response. This was indicated in the experiments of GRAY and MALCOLM (1951) in which it was found that a conditioning stimulus of long duration might contribute up to 35 % of the threshold to a superimposed test stimulus. In the present investigation an increase n excitation was seen in the less rapidly adapting receptors in the form of an increase of the frequency and the duration of the discharge when the stimulus was repeated after djustment of the stimulator so as to give a preset steady level of displacement. This increase in excitation was graded and parallelled the amplitude of the preset displacement The same type of excitatory effect can be produced by horizontal stretching of the skin as shown by LOEWENSTEIN (1965 a) A steady stimulus may thus produce a heightening of the excitatory state although it is insufficient to maintain it at a suprathreshold level. This will be true during physiological standard conditions such as in the present experiments in which the routine was to avoid any deformation prior to stimula

tion. It is interesting, however that with a high degree of stretch, which is probably not physiological, the adaptation may be counteracted to such an extent that a stationary discharge is obtained from the previously rapidly adapting touch receptors (LOEWENSTEIN 1956 a)

The various signs of enhanced excitability during steady deformation show that mechanical recovery of the receptive structures, if it occurs within the deformed skin, is at least incomplete and may not be the only basis for the rapid adaptation of these receptors (cf. DUN and FEBLEY 1938, LOEWENSTEIN 1956 a, GRAY and SATO 1953 and EYZAGUIRRE and KUFFLER 1955) This may also be due to rapidly declining responsiveness of the mechano-sensitive structure while it is still in a deformed state, to high accommodation in the axon ending supplying the receptor (cf. GRAY and MATTHEWS 1951) or to chemical inhibition (HOAGLAND 1936) The present experiments have not given any direct information about the mechanism behind the adaptation. The behaviour of the receptors during the moving phase and the plateau phase of the stimulation suggest, however that a dual mechanism, or at least two different properties, may be involved both in excitation and adaptation. One mechanism or property may be responsible for the initial discharge and another for the discharge or subliminal change of excitability during the steady phase of the stimulation. A comparison may be drawn with the dynamic and static components of the discharge from muscle spindles (KATZ 1950) In these receptors the mechanism or property responsible for the static component is naturally prevalent, while in touch receptors, whose primary task is to signal rapid superficial displacements rather than tension, it seems to manifest itself mainly under special circumstances such as high stretch (LOEWENSTEIN 1956 a) or changes in the chemical environment (TALAAT 1933

The critical slope was between 0.8 and 40 mm/sec for one third of the receptors and for the rest it was of or below 0 8 mm sec. These values are of the same order of magnitude as those obtained by GRAY and MALCOLM (1951) although different techniques have been used. Gray and Malcolm, working on nerve-skin preparations, applied their stimuli to the inside of the skin so as to stimulate the termination of a single axon, while in the present investigation the receptors were studied in situ and the stimuli were applied to the skin surface over individual receptors. In their experiments Gray and Malcolm also measured the threshold at various stimulus gradients and the curve shown by them (GRAY and MALCOLM 1951 Fig 5) follows the same course as that in our Fig. 4

It has been shown that the threshold does not decrease progressively with increasing gradient of the stimulus except for gradients near the critical slope. This fact, which may be looked upon as a limitation of the receptor to respond to rapid stimuli, may be due to inertia of the tissue through which the mechanical forces have t be transmitted or of the receptor itself. It is known, however that the minimum latency of the initiation of an impulse in these receptors is very short according to GRAY and MALCOLM (1951) t is of the order of

1 msec. This and other circumstances, such as the rapid recovery of the skin (LINDBLOM 1958) and the effectiveness of vibratory stimulation (CATTELL and HOAGLAND 1931) indicate that the inertia is slight and that the receptor is capable of reacting rapidly. The relative insensitivity to variations in the rate of deformation may therefore be an inherent property of the receptor which has no simple physical explanation. It may be the axon ending in the receptor that is responsible for the insensitivity. TASAKI (1950) has shown that the same type of threshold-gradient curve is obtained on electrical stimulation of the toad's nerve fibres.

On *suprathreshold* stimulation an increase of the gradient was accompanied by a shortening of the latency and an increase of the frequency of the discharge. Concomitantly the duration of the discharge was reduced, mainly because an increase of the gradient alone means that the effective moving phase of the stimulus becomes shorter. This sequence of changes will be generally valid if it is assumed that the amplitude of stimuli occurring in natural, as well as experimental, situations has to be limited. On stimulation with a steep gradient the adaptation will have relatively less time to exert its action and this would seem to result in a relatively greater total number of impulses. This may not be true, however, because the refractory state summates during repetitive discharge and becomes a more powerful limiting factor the higher the firing frequency tends to be (cf. LINDBLOM 1958). The ultimate frequency and duration of the impulse discharge, therefore, will be dependent upon the resultant action of the characteristics of the stimulus and the various excitability properties of the receptor and axon ending.

The temporal fluctuations of the critical slope, as well as the similar fluctuations of the threshold (LINDBLOM 1958), will diminish the exactness with which outer stimuli will be reproduced, since the afferent discharge elicited by identical stimuli will vary slightly in an unpredictable way. The temporal fluctuations may be random variations in excitability, but they may also represent an integrative mechanism, which would be a centrifugal regulation of the sensory input at the receptor level (cf. LOEWENSTEIN 1956 b). It may be mentioned here that there are also spatial variations in excitability within the sensory unit, since the various receptors supplied by the same afferent fibre usually have different thresholds (LINDBLOM 1958). In comparison with the temporal and spatial variations in excitability, the limited capacity of the receptor to follow changes in the characteristics of the stimuli, as e.g. the relative insensitiveness to variations in the gradient, may be subordinate.

All receptors have been classified as low threshold rapidly-adapting receptors, but it should be pointed out that there are wide variations in the rate of adaptation and the threshold from one receptor to another which are far outside the range of the temporal fluctuations. All intermediate forms seem to exist between the very rapidly and the less rapidly adapting receptors, as well as between those with a relatively high and a relatively low threshold. It is possible that the

most rapidly adapting receptors with a critical slope of about 100 rheobases per second or more (cf. table 1) constitute a subgroup which should be separated from the rest of the receptors. This would be in line with the findings of DUN and FINLEY (1938) and FESSARD and SEGERS (1942) and also with the impression gotten by MARUHASHI, MIZUGUCHI and TASAKI (1952) that there are two kinds of tactile end-organs in the toad's skin, one "phasic" and one "semitonic". A close comparison with the classification proposed by other authors is not possible since different techniques and criteria have been used. Several selective factors are also involved. Thus, in the present material one such factor is the dissection, which may have led to an over-representation of fibres resistant to mechanical injury and another that only units innervating the lower leg have been included. Further the receptors having a relatively high or a very low threshold, or a critical slope below 0.8 mm/sec, could only be subjected to an incomplete analysis. As far as can be judged these receptors have the same general properties as those which could be studied through the whole range of slopes and amplitudes of displacement. As yet, however it cannot be decided, at least as far as the toad is concerned, whether there are any subgroups of functional significance within the population of tactile units with coarse afferent fibres.

The critical slope for discharge and the duration of the discharge on suprathreshold stimulation may be taken as two different measures of adaptation. It would be interesting to compare these two data to see how close the correlation is. Although the technique we have used makes such a comparison possible the material so far analyzed is too small to allow any conclusion in this respect. Nor can it be decided whether the critical slope is correlated to the rheobase value.

References

ADRIAN, E. D., McK. CATTELL and H. HOAGLAND, Sensory discharges in single cutaneous nerve fibres. *J. Physiol. (Lond.)* 1931. *72*. 377—391.

ADRIAN, E. D. and Y. ZOTTERMAN, The impulses produced by sensory nerve endings. *J. Physiol. (Lond.)* 1926. *61*. 465—483.

CATTELL, McK. and H. HOAGLAND, Response of tactile receptors to intermittent stimulation. *J. Physiol. (Lond.)* 1931. *72*. 392—404.

DUN, F. T. and C. B. FINLEY, The rate of adaptation of cutaneous nerve endings in the frog. *J. Physiol. (Lond.)* 1938. *94*. 170—176.

EYZAGUIRRE, C. and S. W. KUFFLER, Processes of excitation in the dendrites and in the soma of single isolated sensory nerve cells of the lobster and crayfish. *J. gen. Physiol.* 1955. *39*. 87—119.

FESSARD, A. and M. SEGERS, Dualité des récepteurs tactiles chez la grenouille. *C. R. Soc. Biol. (Paris)* 1942. *136*. 666—667.

GRAY, J. A. B. and J. L. MALCOLM, The excitation of touch receptors in frog's skin. *J. Physiol. (Lond.)* 1951. *115*. 1—15.

GRAY, J. A. B. and P. B. C. MATTHEWS, A comparison of the adaptation of the pacinian corpuscle with the accommodation of its own axon. *J. Physiol. (Lond.)* 1951. *114*. 454—464.

GRAY, J. A. B. and M. SATO, Properties of the receptor potential in pacinian corpuscles. *J. Physiol.* (Lond.) 1953. *122.* 610—636.

HAAPANEN, N. L. E., unpublished data 1960.

HOAGLAND, H., On the mechanism of adaptation of mechanoreceptors, *Cold Spr. Harb. Symp. quant. Biol.* 1936. *4.* 347—357.

HÖGLUND, G. and U. F. LINDBLOM, Quantitative studies on excitability in touch receptors. *Acta physiol. scand.* 1960. *50.* Suppl. 175. 74—75.

KATZ, B., Depolarization of sensory terminals and the initiation of impulses in the muscle spindle. *J. Physiol.* (Lond.) 1950. *111.* 261—282.

LINDBLOM, U. F. Excitability and functional organization within a peripheral tactile unit. *Acta physiol. scand.* 1958. *44.* 1—84.

LOEWENSTEIN, W. R., Excitation and changes in adaptation by stretch of mechanoreceptors. *J. Physiol.* (Lond.) 1956 a. *133.* 588—602.

LOEWENSTEIN, W. R., Modulation of cutaneous mechanoreceptors by sympathetic stimulation. *J. Physiol.* (Lond.) 1956 b. *132.* 40—60.

MARUHASHI, J., K. MIZUGUCHI and I. TASAKI, Action currents in single afferent nerve fibres elicited by stimulation of the skin of the toad and the cat. *J. Physiol.* (Lond.) 1952. *117.* 129—151.

TALAAT, M., The effect of ions on the cutaneous sensory endings of the frog. *J. Physiol.* (Lond.) 1933. *79.* 500—507.

TASAKI, I., Electrical excitation of the nerve fiber by linearly increasing currents. *Jap. J. Physiol.* 1950. *1.* 1—6.

ZOTTERMAN, Y. Touch, pain and tickling: an electro-physiological investigation on cutaneous sensory nerves. *J. Physiol.* (Lond.) 1939. *95.* 1—28.

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A Presynaptic Effect of d-Tubocurarine in the Neuromuscular Junction

By

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Abstract

LILLEHEIL, G. and K. NAESS. *A presynaptic effect of d-tubocurarine in the neuromuscular junction.* Acta physiol. scand. 1961. 52. 120—136. — End-plate potentials (e. p. p.) of isolated phrenic nerve diaphragm preparation have been recorded at different stages during development to complete curarization and during recovery of the preparations. The first effect obtained during increasing curarization is characterized by the constancy of the e. p. p. during tetanic stimulation, i.e. by lack of Wedensky inhibition. This phenomenon, with a rapid decline of e. p. p., develops under the influence of tubocurarine and must probably be caused by this pharmacological influence. It is concluded that the mechanism behind the Wedensky inhibition must be of presynaptic origin, and probably be due to a reinforcement of the naturally occurring tendency to block of propagation of tetanic impulses in the last arborization of the motor nerve.

The following topics are more comprehensively treated in the discussion. 1. The possible presynaptic effect of tubocurarine in relation to the classical theory of a pure postsynaptic one demonstrated by DALE, FELDBERG and VOGT (1936) when single stimulation were used. 2. The possible course of release of acetylcholine during tetanic stimulation. 3. Possible desensitization of receptor substance to ACh during transmission of repetitive impulses. 4. Effect of other quaternary ammonium compounds on nerve fibres, especially on unmyelinated nerve endings.

Since the work of DALE, FELDBERG and VOGT (1936) the effect of (curarine) d-tubocurarine has been believed to be due to a pure postsynaptic mechanism — the competition between acetylcholine (ACh) and the drug for the receptor

substance. These authors found no change of output of ACh under the influence of curarine. Tetanic stimulation was, however not used in this investigation and the work gave, therefore, no clue to the understanding of the mechanism behind the Wedensky inhibition — a phenomenon so characteristic for the effect obtained by curarization.

Since 1936 a series of papers on the effect of tubocurarine and investigations in which this drug has been used as a tool have been published. Tetanic stimulation has, as a rule, been used in these publications and the discussions and conclusions have generally been based on the assumption that curare produces no change of output of ACh even during tetanic stimulation. This assumption has, however never been proved. Some papers mentioned in the discussion have, on the other hand, given some indication of an additional presynaptic effect of tubocurarine, but these papers have never received much attention.

The purpose of this investigation has been to submit this possible presynaptic effect of tubocurarine to a closer investigation. The main result of the investigation is that the mechanism behind the Wedensky inhibition produced by tubocurarine must be of predominantly — if not exclusively — presynaptic origin. Our findings are comprehensively discussed in relation to relevant information concerning the mechanism for synaptic transmission, and particular attention is paid to the question of variation of release of the transmitter under physiological conditions.

A preliminary report of our results has been published recently (LILLIESTRAND and NASTUK 1960)

Methods

Isolated rat phrenic nerve diaphragm preparations have been used. Action potentials have been recorded with two electrodes, one in the central part of the motor end-plate region and the other few mm away on the same fibres. The first one records the end-plate potential (e. p. p.) under normal conditions with diphasic action potential superimposed, and after appropriat curarization pure monopolar end-plate potential, while the other one records spike potential from the muscle fibres outside the neuromuscular junction. The potentials are recorded with the preparation submerged in bath of oxygenated (+ 5 % CO_2) Tyrode solution. Rectangular supramaximal stimuli of 0.5 msec were applied to the phrenic nerve, either as single impulses with frequency of 1/sec or as tetanic stimulation with a frequency of 50/sec, which is believed to be approximately the maximal naturally occurring frequency in this preparation. The traces on the cathode ray screen have often been blanked for time intervals of 1/3 sec under tetanic stimulation to have the potentials clearly separated for publication. Continuous registration on film has also been used.

Location of the end-plate zone is a relatively simple procedure when the following method and criteria are used 1 The macroscopically visible end-plate zone observed in the partly transparent preparation transilluminated from below 2. The shortest latency between the stimulus artefact and the start of the potential, and the characteristic change of shape of the potentials when passing the electrode across the end-plate zone and the adjacent region. 3. The typical monopolar potential obtained after complete

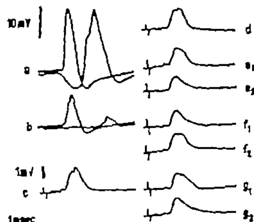


Fig. 1 Action potentials from rat diaphragm preparation after indirect, supramaximal stimulation of phrenic nerve) Single stimulation before curarization. First potential from end-plate zone, second potential from the muscle fibre few mm away from this zone. b) 8 min after addition of 3 µg/ml d-tubocurarine chloride to the bath.) Potential from end-plate zone after single stimulation 14 min after curarization. Note increase in amplification for this and the following recordings. Muscle fiber action potential has now disappeared, and the beam for recording of this potential has been switched off. d—g) Some of the action potentials recorded with the same electrode as in c during tetanic stimulation (50/sec) immediately following c. d = First potential, , and = after 1 sec. f and f_2 = after 8 sec. g_1 and g_2 = after 16 sec.

curarization 4 As an additional control, the highest potential after disappearance of the spike potential.

d-T bocurarine chloride ("Nyco") has been added to the bath. The term complete curarization is in this paper used for a stage when the spike potential has disppeared, leaving the local non-propagated potential in the end plate zone.

Results

The results are presented in 3 figures, the two first ones demonstrating in their purest form the two typical effects obtained at different stages of curarization. The third demonstrates more continuously the development of the effect after addition of tubocurarine and during recovery of the preparation

The first recording in Fig 1 demonstrates the normal potentials before curarization, the second one the potentials just before complete curarization

The last recording in the first column is only the potential in the motor end-plate zone when the superimposed spike potential is on the verge of disappearing The potential recorded is now predominantly a pure monopolar e. p. p The second column represents recordings of e. p. p from the same place during tetanic stimulation with 50 st./sec immediately after the single stimulation. It is obvious that the e p p is maintained on an approximately steady level even during a long tetanic stimulation A typical effect of this kind can only be obtained in relatively few preparations during the introductory phase of the effect of tubocurarine and only when an appropriate concentration of tubocurarine has been used for the preparation under investigation. A steady level of the e. p p. can under such circumstances be obtained from approximately 5—15 min after addition With higher concentrations, or after a longer period of action of tubocurarine the potentials are reduced more or less during the

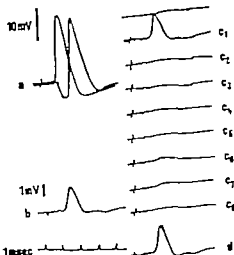


Fig. 2. Similar potentials as in Fig. 1) As in Fig. 1 a. Between and b addition of 0.2 μg/ml d-tubocurarine chloride. Disappearance of muscle fiber action potential after 14.5 min and complete disappearance of end-plate potential after 18.5 min. Washing out of tubocurarine 33.5 min after curarization. b, c, and d recorded 15 min later during recovery of the preparation from the complete block, just before the first muscle fibre potentials reappeared. b) Potential from the end-plate zone after single stimulation. c_1—c_8) Some of the end-plate potentials recorded with the same electrode as in b during tetanic stimulation (50/sec) immediately following b. c_1 first potentials and the other with intervals of 1/3 sec. d) As in b, 3 sec after determination of tetanic stimulation. Note increase in amplification between and b.

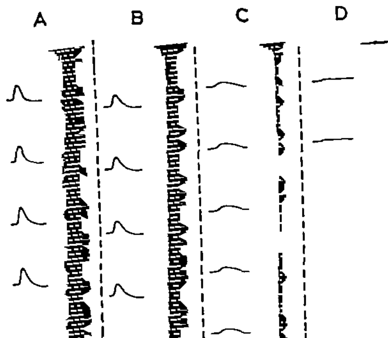


Fig. 3. Changes of p. p. during tetanic stimulation with 50/sec under increasing curarization (0.2 μg/ml d-tubocurarine) and during recovery of preparation. Continuous recording of e. p. p. downwards along the film combined with simultaneous registration of some of the same e. p. p. across the film with intervals of approximately 0.5 sec. A: On the verge to complete curarization. B and C During increasing curarization, D During recovery after complete disappearance of all e. p. p. and washing out of tubocurarine. Relative amplification A and B 1 C 1.3 and D 2.3.

tetanic stimulation and represent then transitory stages to the other typical effect illustrated in Fig 2

Very often an overlapping between the two typical effects demonstrated in Fig 1 and Fig 2 has already developed when the stage of "complete curarization" is obtained, and quite constant potentials can, under these circumstances, not be recorded during tetanic stimulation. The typical picture demonstrated in Fig 2 is also not a quite constant finding, but a rapid decline of the e. p p always takes place in this phase, even if the potentials do not disappear *so rapidly as in Fig 2*

The development in another characteristic experiment can be followed more closely in Fig 3 which requires no other comment than the information given in the legend.

Discussion

The Wedensky inhibition of tetanic contractions has always been considered a typical effect of tubocurarine and substances with similar action.

WEDENSKY demonstrated in his comprehensive work from 1903 the inhibition of conduction and transmission of impulses produced by repetitive stimulation with higher frequencies. He, and also HOFMANN (1903) proved that the neuromuscular transmission was more sensitive to this inhibition than the nerve and the muscle fibres. This naturally occurring limiting factor could be strongly enhanced by different types of physical and chemical influences. In the motor end-plate the inhibition was increased by ether nicotine and curare. All types of inhibition produced by high frequency stimulation have since been called Wedensky inhibition.

Wedensky inhibition of tetanic contraction is usually defined as the phenomenon that these contractions are transformed to very short-lived ones, which are only recorded as a twich by usual mechanical recording This is type of contraction seen during relatively deep curarization of a certain duration (see NAESS 1952 and 1953) but slighter forms of inhibition are also sometimes named Wedensky inhibition.

Since the work of DALE, FELDBERG and VOGT (1936) the mechanism of action of curare has been considered an established fact. The competition between the released acetylcholine and the drug for the receptor substance has been believed to be the whole explanation of the inhibition of transmission, without any interference with the presynaptic production mobilization or release of the transmitter During the last decade the effect of a series of substances of different types has been traced to the presynaptic fibres and/or the mechanism of acetylcholine release. Papers demonstrating this mechanism of action are mentioned at the end of the discussion. Before the final discussion some topics of more general and principal interest must be surveyed and discussed

1 *Discussion of the work and conclusions of* DALE, FELDBERG *and* VOGT

In the description of their method these authors give valuable information concerning certain procedures which may limit the possibilities of too far

reaching conclusions concerning the mechanism of action of tubocurarine. This technical information has never been paid sufficient attention.

It is stated that acetylcholine could only be obtained in the perfusion fluid during stimulation when saline solution was used. When animals were pretreated with eserine and atropin and perfused with blood, no acetylcholine could be found. A certain possibility of working with anoxic muscles must exist when saline solution is used, especially when the muscle is stimulated for longer periods. The neuromuscular transmission and the release of acetylcholine is probably most easily affected by lack of oxygen (WEDENSKY 1903 NAESS and STORM MATHISEN 1955)

DALE *et al.* mentioned that the artificial conditions of the saline perfusion in some way facilitate the escape of acetylcholine from the site of its origin into the blood vessel. FELDBERG SHUL and KARLIN (1936) found that acetylcholine appeared even when the preparation was perfused with blood, if only the blood was not saturated with oxygen. It must be generally accepted that preparation on the verge of anoxia is not the best one for more complete elucidation of all details in the mechanism of drug action, even if the main features of the action may be correctly demonstrated. (For further discussion of the limited significance of ACh appearance in junctional perfusate, see NACHMANSOHN 1959 and section 4 in this discussion.)

DALE *et al* also mentioned that the use of saline solution caused limitation of the experimental period in which success was possible If the period was too long the muscle became *quickly* insensitive to motor nerve impulses. There is, therefore reason to believe that the experiments must have been done in a limited period and that the effect of curarine was tested as soon as the transmission had been blocked after the injection of the drug Previous work (NAESS 1952) and the present results demonstrated that the complete effect of tubocurarine, including what we believe is a presynaptic effect, takes considerably longer time to establish itself than the rapidly occurring postsynaptic one. Experiments of short duration may therefore, not reveal all the effects of tubocurarine.

A factor of central significance will be mentioned at the end of this section Tetanization was not used in the work of DALE *et al* The frequency of stimulation never exceeded 15/sec and single contractions down to 5/sec were often used. "This method, causing a rhythmic series of twitches, was chosen in preference to tetanization, so as to avoid impediment to the perfusion during activity of the muscle." These frequencies cannot — especially during introductory stages of curarization — produce any Wedensky inhibition, and the mechanism behind this phenomenon has, therefore, not been investigated by DALE, FELDBERG and VOGT This has, however obviously been assumed by several other workers.

2. *Previous literature dealing with the release of transmitters during repetitive stimulation*

A chapter dealing especially with the ACh release in the motor end-plate during transmission of repetitive stimuli is necessary for the further discussion. Possible changes in the ACh-production in a short interval at the beginning of such transmission can not be directly measured (see p. 130) This is, however the period of importance in connection with the Wedensky inhibition. It is, therefore, of interest to look at the characteristic features of blocks produced by other procedures than curarization. The lack of Wedensky inhibition of the

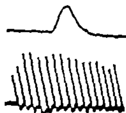


Fig. 4. E. p. p. produced by single stimulation and a short tetanic stimulation with 50 sec after block of transmission by addition of $6 \cdot 10^{-3}$ M $MgSO_4$ to the bath. (Single and repetitive e. p. p. recorded with different time base.)

reduced tetanic contractions of tibialis ant. muscle of cat when depolarizing substances are used, indicates a relatively constant release of ACh per impulse. This assumption cannot be tested in rat diaphragm preparation because the effect of depolarizing substances is so rapidly transformed to a curare like effect with a pronounced Wedensky inhibition. Procedures which reduce the release of ACh, excess of magnesium, lack of calcium and use of botulinum toxin, produce inhibition which is characterized by the opposite phenomenon of a Wedensky inhibition, *i.e.* facilitation through a block. Tetanic contractions increase their amplitudes with increasing frequencies of stimulation, and no drop in amplitude occurs during the stimulation.

Recording of e. p. p. under such circumstances reveals the facilitation as demonstrated by use of magnesium in this paper (Fig 4) This implies a progressive increase of the ACh release per impulse during the first period of stimulation. Block produced by excess of sodium gives no Wedensky inhibition, and a slight increase of e. p p or stable e. p p are obtained by this procedure. Low sodium block also gives constant amplitude of e. p. p. during the first period of stimulation (See section 3)

Inhibition of neuromuscular transmission undoubtedly plays a role in the reduction of more protracted tetanic contractions produced under physiological conditions. Such contractions reduce or completely stop the circulation through the muscle simultaneously with an increased requirement of oxygen. The asphyxia depresses the transmission and is the limiting factor for the strength and duration of the maximal tetanic contraction (For a closer discussion see WEDENSKY (1903) NAESS and STORM MATHISEN (1955).)

The question of interest in connection with our present problem and work is, however whether a sufficient and rapid reduction of ACh output per impulse also takes place during tetanic contractions of short duration, *e g* up to 5 sec. This is absolutely necessary if the Wedensky inhibition seen under curarization shall be explained by the generally accepted theory of ROSENBLUETH and MORISON put forward in 1937 Their suggestion of a rapidly declining ACh-production during transmission of tetanic impulses, at that time, based on predominantly hypothetical considerations. The following authors have up to the present time, been repeatedly quoted in support of the above mentioned theory BROWN (1937) ECCLES KATZ and KUFFLER (1941) LILEY and NORTH (1953)

BROWN states in his paper: "BROWN and DALE (1937) have evidence that in a succession of nerve impulses each successive impulse liberates progressively less of transmitter if they are sufficiently close together." This refers to unpublished results, which do not allow closer evaluation. We do not know the frequencies used to obtain a reduction, or whether the investigation — like the following ones — has been made under curarization, which with our present knowledge must be considered a serious objection against this statement.

ECCLES *et al.* and LILEY and NORTH have used curarized preparations. The fact that the e. p. p. decreases during tetanic stimulation in curarized preparations cannot, however, on the following grounds give any certain information of the processes under normal conditions. All authors who have taken this reduction as a proof of a progressive decline of the ACh-production have accepted the statement of DALE *et al.* of a pure postsynaptic effect of curarine as an absolute fact. As stressed earlier, DALE *et al.* have, however, never made any investigation of the ACh-release during tetanic contractions (see section 1).

HUTTER (1953), who made well-founded investigation of the facilitation and inhibition of the neuromuscular junction, was of the opinion that his results had proved the hypothesis of ROSENBLUETH and MORISON (1937). He demonstrated that the ACh-sensitivity of the motor end-plate did not change during a Wedensky inhibition. He consequently concluded that the Wedensky inhibition must be due to a pure presynaptic mechanism of action. We certainly agree in this conclusion, but cannot accept the result as a proof of a naturally occurring decrease of ACh production under physiological conditions as suggested by ROSENBLUETH and MORISON. HUTTER's conclusion is, thus, also based on the statement that tubocurarine has no presynaptic effect, an assumption which is never proved and — we believe — disproved by our present work.

The experimental results of HUTTER are, however, of great value and form a cornerstone in our main discussion (section 5).

KRNJEVIC and MILEDI (1958) have made a comprehensive study of the neuromuscular block produced by long sustained tetanic stimulation with different frequencies. Their result has also been quoted as support of a theory of pre-junctional deficiency in the release of the transmitter substance. Their experiments gave, however, no proof of an exhaustion of ACh as the mechanism behind the inhibition of transmission obtained in their experiments.

Microelectrodes were introduced intracellularly during tetanic contractions which, as a rule, had gone on for a long period before registration of e. p. p. was started. This investigation can, therefore, give no information of a possible naturally occurring deficiency in the release of the transmitter in the first period of transmission of tetanic impulses. The very reliable results of these authors demonstrate that two types of block are at work during long sustained tetanic contractions, a presynaptic failure of propagation and another of postsynaptic origin. These types of block develop after a relatively long period of stimulation, and are irrelevant in connection with our present problem. The relatively low safety factor for conduction in the unmyelinated presynaptic fibres is, however, of special interest in connection with our final discussion of the mechanism behind the presynaptic effect of tubocurarine (section 5 b).

The conclusion of this survey of previous relevant literature must therefore be. There is no direct or indirect proof of a rapid and progressive reduction of the ACh release per impulse during transmission of tetanic impulses, i.e. no proof of a naturally existing basis for the Wedensky inhibition, which is especially characteristic for curarization.

The increase of e. p. p. in the beginning of a tetanus in frog during curarisation (Eccles et al. 1941) is also worth mentioning. There must probably be an increase in the release of ACh per impulse in this preparation and no rapid depletion as assumed for the mammalian preparations. We believe that the difference observed by curarization of the two types of preparation is due to a more slow presynaptic effect of the drug in frog. This would make the pure postsynaptic effect of curare more readily observable in this species of animal.

It may be of interest to look for other processes of transmission, which can be observed in more detail without interference by any drug. R. Eccles (1955) has recorded synaptic potentials by microelectrodes in ganglia of rabbits. The stability of these potentials, even with relatively higher frequencies (up to 40—50/sec) was remarkable in this investigation too. No significant decline in the amplitude took place in the beginning of the transmission of repetitive stimuli and there seems therefore, not to be any decrease of ACh release in this transmission when limited periods of stimulation were used.

The stability of the postsynaptic excitatory potentials recorded from the motoneurons of the spinal cord during high-frequency stimulation of afferent fibres has been demonstrated by Curtis and Eccles (1960). These authors assume a general mechanism for different types of transmission by which each impulse causes a depletion of transmitter simultaneously with a mobilisation of new transmitter from the store. With somewhat higher frequencies of stimulation the last process may compensate or even overcompensate the first one. The following is stated with regard to the motoneurons:

In this way high frequency of synaptic activation calls forth a mobilisation of transmitter that ensures a greater synaptic effect veneni for rates of synaptic activation up to 250/sec. An increase may even be seen in the excitatory synaptic potentials recorded intracellularly by microelectrodes with frequencies from 80—125/sec. The release of the transmitter in these synapses must obviously have a high safety factor with respect to transmission of repetitive stimuli.

It is not easy to understand why motor end-plate should be an exception with a *rapidly* declining release of transmitter under physiological conditions, and we are — as already stated — of the opinion that a series of direct evidence seems to disprove this assumption. (See also addendum.)

5. Desensitization of the motor end-plate to ACh during tetanus

Another hypothesis put forward by Thesleff (1959) which could partly explain the Wedensky inhibition must also be mentioned and briefly discussed. Thesleff used hypertonic NaCl solution to block the neuromuscular transmission and found a relatively slight reduction of e. p. p. combined with a sometimes strong and rapid reduction of the sensitivity of the motor end-plate to electrophoretically applied ACh. The consistent conclusion was the assumption of a rapid desensitization of the motor end plate to ACh during tetanus. This could, according to Thesleff, partly explain the mechanism behind the

phenomenon of Wedensky inhibition. Some of his findings and the hypothesis have been tested and discussed by the present authors in a special paper (NASTS and LILLEHEIL 1961). The main result of this paper was the constancy of amplitude of e. p. p. during a tetanus recorded immediately after the preparation was blocked by excess of sodium. Mechanical recordings of tetanic contractions during increased NaCl concentrations did not show any sign of Wedensky inhibition either. THESLEFF's observation made with the addition of NaCl can, therefore, probably not explain the mechanism behind the phenomenon of Wedensky inhibition. The reader is referred to the paper of LILLEHEIL and NASTS (1961) for a more comprehensive discussion. Three other experimental results which are not in agreement with the hypothesis put forward by THESLEFF however will be especially mentioned here.

HUTTER (1953) tested the ACh-sensitivity of the motor end-plate to ACh before and during a completely Wedensky-inhibited tetanic contraction in a curarized preparation. He found no change in the sensitivity and concluded that such a mechanism could not explain the inhibition.

Quite recently OTSUKA and ENDO (1960) using microelectrodes and electrophoretically applied ACh in curarized frog preparations confirmed this result and could not support the finding of THESLEFF indicating a rapid desensitization of end-plate to ACh. LILEY (1956) observed the miniature potentials during and immediately after transmission of tetanic impulses and found no change in the amplitude of these potentials. Once again the conclusion must be drawn that probably no change in the ACh-sensitivity occurs during the first part of tetanic stimulation under physiological conditions, even if this is the case during more prolonged tetanic stimulation (KATZ/KRNJEVIC and MILEDI 1958).

4. *Main discussion of present results*

The stability of the e. p. p. in the first seconds of tetanus in the first phase of curarization must, according to the present results, be considered a fact.

It is not possible to obtain quite constant amplitude in the introductory phase in all preparations, but the decline is not more than about 20 per cent in 10—15 sec when the correct concentration of tubocurarine and the correct time for registration are chosen. There are in this respect certain individual variations dependent on the age of the animal and thickness of the preparation. The last factor is of significance for obtaining a rapid and even effect in all fibres from which the extracellular electrode records.

The Wedensky inhibition of the e. p. p. *i.e.* the rapid decline in the potentials, develops progressively under the influence of tubocurarine, but is to a certain extent covered by the simultaneous reduction of all potentials. The rapid decline of the e. p. p. is, therefore, most readily observed during the recovery of the preparation after tubocurarine has been washed out. The unchanged ACh-sensitivity of the motor end-plate during tetanic stimulation (see section 2 and 3) entails that the relative inhibition of e. p. p. produced during tetanus must be considered to be of presynaptic origin and the result of the experi-

mental procedure i.e. curarization. *Tubocurarine must have produced this effect itself in its second phase of action.* This conclusion is the same as stated previously from results obtained by mechanical recordings of muscular contractions in rabbits (NAESS 1952, 1953)

The different time course of production of the pre- and postsynaptic effects, which make the separation of the two mechanisms possible, must probably be due to different penetrability of tubocurarine into the two structural membranes concerned — *the postsynaptic membrane being the most readily affectable.*

It has previously been demonstrated that the postsynaptic effect reaches its maximum 3—4 min after intravenous injection in rabbits and the possible presynaptic effect is fully developed after 15 min when continuous injection is used (NAESS 1952) It is not so easy to give exact information concerning the time factor when rat preparations are used due to the more pronounced overlapping of the effects when the drug must penetrate into the preparation and thus is administered more slowly and at various speeds to the different end-plates.

The finding that the Wedensky inhibition is most distinctly observed during the recovery of the preparation (see Fig 2 and 3) is according to our opinion probably due to the more rapid removal of tubocurarine from the postsynaptic bindings. This will reduce the competitive postsynaptic inhibition more rapidly than the presynaptic one, which is responsible for the Wedensky inhibition.

A direct measurement of the ACh-output before and during curarization may perhaps, seem necessary to substantiate our postulate of a presynaptic effect of tubocurarine. We will, therefore, explain the reason why we do not rely on this method when tetanic stimulation is used

The difficulties encountered by DALE *et al.* with perfusion of muscles *in vivo* (see section 1) are almost an adequate explanation. Use of isolated preparation and examination of the output of ACh in the bath during long sustained tetanic stimulation may seem to be a more reliable method. Long sustained tetanic stimulation necessary to produce sufficient amounts of ACh in the bath is, however a questionable method, because it causes the neuromuscular junctions to work under unphysiological conditions, which to a certain degree are similar to those produced when the muscle is perfused and stimulated *in situ.* (For further discussion of significance of variation in measurable ACh see NACHMANSOHN (1959) pp. 206—208.)

Addition of inhibitors of cholinesterase to the bath is necessary The ACh produced is not split, and lack of choline with significant influence on the restoration of the presynaptic store of ACh may ensue. Excess of ACh around the presynaptic fibres can certainly interfere with the possibilities of other quaternary ammonium ions, e.g. tubocurarine, to interfere with the activity of the presynaptic fibres (see different presynaptic effect of quaternary ammonium ions p. 152 and possible presynaptic effect of ACh mentioned in the last section of the discussion (section 5) Inhibitors of cholinesterase and structurally related substances seem by themselves to have certain presynaptic effects (see RIKER *et al.* 1957 RIKER *et al.* 1959, NACHMANSOHN 1959 p 198) which may interfere with the transmission of tetanic impulses and produce unphysiological basic conditions for this type of experiment.

It is also of interest to mention the recently demonstrated effect of adrenergic blocking agent on the "overflow" of noradrenaline into the perfusion fluid (Brown

1960) and the hypothesis of presynaptic effect of this type of drugs put forward by PATON (1960) in the discussion of this result.

A series of arguments thus limit the reliability of results obtained in this way and we found no reason to perform experiments of this type to support or disprove our theory Direct measurement of the e. p p., combined with relevant information from other reports is, in our opinion, a far better and more trust worthy procedure, especially with regard to the first short interval of transmission of impulses — the period of interest for the actual problem — the mechanism behind the Wedensky inhibition in curarized muscles.

A series of previous results have given certain indications of a presynaptic effect of tubocurarine. In a few instances a theory including a presynaptic effect of tubocurarine has been proposed on the results obtained by ARDOIN and BJARKE (1945) NARDI (1952, 1953) and the last time quite recently by RIKER *et al.* (1959)

Relevant papers are briefly quoted here

BOCHTRAL and LINDHARD (1942) investigated the function of isolated motor end-plates of the lizard. Though they demonstrated the specific sensitivity of the motor end-plate to ACh, and also showed the reduction in this sensitivity produced by curarine, they found by means of electrical stimulation that the first boundary of the motor end-plate, the neurogenic component, was blocked at time when the excitability of the myogenic component, the second boundary was not changed. After acting for some time, however curarine also blocked this boundary

ENGBAEK (1948) has repeated this experiment and confirmed the results. She gives information on the concentration necessary to block the two components of the motor end-plate. ENGBAEK mentions that the results suggest blocking of terminals of the motor nerve but dismisses this possibility owing to the then accepted fact that curare does not influence the activity of peripheral nerves even t high concentrations. (See however DETTBARN (1960) GRUNDFEST and NACHMANSOHN (1952))

ARDOIN and BJARKE (1945) by a biochemical method, found no production of free acetylcholine after indirect tetanic stimulation of a frog muscle, while direct stimulation still produced normal amounts. The method of these authors has, however been criticized and it has been difficult to use in the hands of other scientists. (V P WHITTAKER and others, personal communication.)

LAPORTE and LORENTE DE NÓ (1950) have demonstrated that an increase in the L-fraction of the membrane of presynaptic fibres of the sympathetic ganglion of turtle takes place during curarization. From earlier observations of LORENTE DE NÓ it is known that an increase in the L/Q ratio of membrane potentials will produce a block which will be reinforced by long trains of impulses. (See also section 5 b.)

NARDI (1952 1953) concluded on the basis of changes in the form of myograms obtained under development of curarization in rabbits that tubocurarine presumably had a presynaptic effect in addition to the postsynaptic one.

RIKER *et al.* (1957) demonstrated that certain phenolic quaternary ammonium ions facilitate neuromuscular transmission primarily by an action on the motor nerve terminals. The presynaptic effect was especially specific for 3-hydroxyphenyl-triethyl-ammonium (3-OH PTEA) It was demonstrated in the same paper that small doses of tubocurarine and also procain well below those that interfere with single-volley transmission, obliterate the repetitive antidromic activity in the nerve produced by 3-OH PTEA. Hence it was concluded that the blocking effect of curare in the motor end-plate occurs

primarily at the motor nerve terminals. It is, for the further discussion, of interest to mention that the repetitive firing produced by 3-OH PTEA and blocked by tubocurarine usually was of high frequency (about 100/sec or above)

Our investigation gives no information which can support the hypothesis of RIKER *et al.* of a *primarily* presynaptic effect of tubocurarine. *The importance of the well-established postsynaptic competitive effect of tubocurarine* (DALE *et al.*) *is of course fully accepted by us. The presynaptic effect, demonstrated in the present paper comes in addition to the postsynaptic one.* The former can be observed especially — or perhaps only — during transmission of tetanic impulses. The higher the frequencies of stimulation, the stronger is this effect. The investigation of DALE *et al.* has forever demonstrated that the postsynaptic effect is the dominant one when single stimuli are used. Nothing in our results is at variance with this well founded and generally accepted investigation. The effect of small doses of tubocurarine on the repetitive discharge of presynaptic fibres produced by special substances — as demonstrated by RIKER *et al.* (1959) has also primarily revealed an inhibitory effect on the ability of production and conduction of repetitive impulses of higher frequencies. The result is, thus, also in agreement with our results and conclusion. We are of the opinion that the indirect evidence of a presynaptic effect, demonstrated by RIKER *et al.* (1959) can not prove anything certain regarding a possible presynaptic effect of tubocurarine on transmission of single impulses under normal conditions. It seems at present, most reasonable to trust the classical work of DALE *et al.* in this respect.

5 *Possible mechanism of presynaptic action of tubocurarine*

Very little is known about the finer mechanism of conduction or electronic spread of impulses of the smallest presynaptic fibres and of the release of ACh. It is, therefore, impossible to give a more detailed theory for the mechanism behind the presynaptic effect of tubocurarine. Some factors of interest in this connection ought, however, to be mentioned here.

a. *Penetrability of quaternary ammonium ions into the nerve fibres especially presynaptic ones*

It was, until quite recently, the general opinion that quaternary ammonium ions due to their low lipid solubility and completely charged state could not penetrate into nerve fibres, including the smallest ones. Different results obtained by various workers during the last three years have, however, demonstrated that the finest terminals of efferent as well as afferent nerves differ from the main part of the myelinated nerve fibre in this respect. STOVNER (1958 a) and KOKETSU (1958) demonstrated independently that tetraethyl ammonium affected the presynaptic fibres and increased the output of ACh per impulse, thereby producing an antiblocking effect on different types of inhibition of the neuromuscular transmission (see also STOVNER (1958 b)). RIKER *et al.* (1957, 1959) demonstrated an ability of special quaternary ammonium ions to produce repetitive discharge in the presynaptic fibres, and RIKER and SZRENIAWSKI (1959) achieved a corresponding effect on the pre

synaptic fibres in the sympathetic ganglion The latter authors were also of the opinion that they could demonstrate a depressive effect of certain ganglionic blocking substances, among others hexamethonium, on these presynaptic fibres.

It has also been shown that juxtaarterial injection of ACh can stimulate the terminals of sensory fibres and this effect can be readily blocked by hexamethonium (DOUGLAS and RITCHIE 1960) The new adrenergic blocking compound, bretylium, which is also a quaternary ammonium ion, acts on the postsynaptic sympathetic fibres, and the strongest effect seems to occur on the nerve endings (GREEN 1960) Even cholinesterase inhibitors of the quaternary ammonium group seem to have certain presynaptic effects (for references see RIKER *et al* 1957 NACHMANSOHN 1959) Additional indications of an effect of quaternary ammonium ions on presynaptic structures can be found in the chapter on previous reports of presynaptic effects of tubocurarine (section 4) We must therefore agree with the statement of RIKER *et al.* (1957) that three possible effects exist for all quaternary ammonium ions i) a postsynaptic one (depolarizing or stabilizing) ii) an inhibition of the cholinesterase and iii) a presynaptic effect which now according to previous and present results, obviously can be of either stimulatory or depressive character

b. *Possible blocking of presynaptic repetitive conduction by tubocurarine*

GRUNDFEST and NACHMANSOHN (1952) and quite recently DETTBARN (1960) have demonstrated a blocking effect of tubocurarine on the axon itself when special methods are used. Relatively high concentrations of tubocurarine are necessary to block the single impulses at Ranvier nodes (10^{-4}M) but it is highly probable that far lower concentrations are capable of producing Wedensky inhibition of conduction without any significant influence of the first impulse in the volley *i.e.* similar to procaine. Still lower concentrations may — due to a greater permeability of the presynaptic fibres — influence the conduction in this structure in the same way producing Wedensky inhibition of conduction of tetanic impulses in the last terminals of the motor nerve

RIKER *et al.* (1957) thus, demonstrated a similar presynaptic effect of small doses of tubocurarine and procain, well below those that interfere with single volley transmission. The most probable presynaptic mechanism of action of tubocurarine seems therefore, to be a reinforcement of the naturally occurring tendency to Wedensky inhibition of conduction in the presynaptic fibres demonstrated by KRNJEVIC and MILEDI (1958)

Our conclusion as to the presynaptic effect of d-tubocurarine in the neuromuscular transmission of mammals is completely in agreement with results of LAPORTE and LORENTE DE NÓ (1950) on the sympathetic ganglion of turtle. They stated in the summary Although tubocurarine produces certain changes in the properties of the ganglion cells curarization is mainly caused by modification in the properties of the presynaptic fibrils, which results in the establishment of blocks of conduction in the presynaptic arborization These authors

also observed different stages of curarization with a qualitative change of the effects and they also worked with repetitive stimulation to reveal the presynaptic effect.

c. *Competition with ACh for presynaptic receptor ?*

BARSTAD (personal communication 1960) has in preliminary experiments demonstrated that ACh itself increases the frequency of the miniature potentials. (See also the conclusion of MASLAND and WIGTON 1940) The naturally released ACh may possibly have the same effect, an action which, according to recent results (ECCLES *et al.* 1959), indicates a depolarization of the presynaptic terminals, *i.e.* a presynaptic potential. The possibility exists that tubocurarine by means of a competitive mechanism can block this effect of ACh, thereby producing a Wedensky inhibition of the release mechanism. A further discussion of this hypothesis would at the present time be premature and is therefore omitted from this paper

It is generally accepted that substances which reduce the release of ACh do not produce any Wedensky inhibition. It seems now however to be necessary to accept two different mechanisms behind a reduction of the output of ACh, each giving a special picture of inhibition. It is in this connection, of special interest to mention quite recent results obtained by CHANG (personal communication) with different fractions of *Bungarus multicinctus* venom. The crude venom has previously been investigated (CHANG 1959 a, b) and a curare-like inhibition of the neuromuscular transmission has been demonstrated. Four different fractions have now been separated, one having a competitive post synaptic effect similar to that of tubocurarine. Two others have no effect on the receptor substance but inhibit the release of ACh. The surprising result is that these substances, in contrast to botulinum toxin, gave a pronounced Wedensky inhibition. These two fractions seem, in our opinion, to exert a presynaptic effect in its pure form similar to that proposed for tubocurarine in this paper

Addendum

STRAUGHAN (1960) has recently published results of his investigation on the release of ACh from nerve terminals in the rat and guinea pig phrenic nerve diaphragm preparation. The paper is of special interest in connection with our present results and considerations.

STRAUGHAN demonstrated an astonishing maintainance of the ACh-production *throughout prolonged periods of stimulation* (20 min). From details in his investigation he drew a similar conclusion as KRNJEVIC and MILEDI (1958) previously mentioned in our discussion: "It seems unlikely that there is any depletion of the acetylcholine stores in the terminals during a tetanic period of stimulation even when there is complete neuromuscular block." STRAUGHAN is of the opinion that the decline in the release of ACh during prolonged tetanic stimulation is due to the mechanism demonstrated by the two above mentioned authors, *viz.* an intermittent presynaptic failure of conduction. STRAUGHAN's results indicate that this intermittent presynaptic failure is absent initially during tetanic stimulation, i. e. in the period in which the Wedensky inhibition during curarization takes place. The usually accepted hypothesis of a rapid decline of the ACh-production is not in accordance with STRAUGHAN's results and conclusion. This author therefore suggests a rapid reduction of the ACh-sensitivity

(a desensitization) of the motor end-plate as a possible explanation of the Wedensky inhibition. This possibility is, as seen in our discussion, rejected due to results obtained by ourselves and other authors (HUTTER 1952, OTSUKA and ENDO 1960).

STRADOUBAN also mentions another possible explanation for the Wedensky inhibition "an enhancement of the blocking activity of curare (CHOU, 1947). CHOU demonstrated that "the effect produced by tubocurarine is greater when the muscle is working and is a function of the rate of stimulation".

The Wedensky inhibition seen during curarization is also a phenomenon characterized by a rapid increase of the block of transmission of impulses of higher frequencies. A pure description of the phenomenon, as found in CHOU's work, cannot give any explanation of the mechanism behind either the decline of the single contractions or the Wedensky inhibition. We are of the opinion that both the well known fact described by CHOU and the Wedensky inhibition are due to the same mechanism, and have in our main paper tried to find a satisfactory explanation, which also fits well with the new results obtained in STRADOUBAN's thoroughly performed investigation of the ACh-production during tetanic stimulation.

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References

ABDON, N.-O. and T. BJARKE, The mechanism of acetylcholine liberation in striped muscles. *Acta pharmacol.* (Kbh.) 1945. *1* 1—17.

BROWN, G. L., Transmission at nerve endings by acetylcholine. *Physiol. Rev.* 1937 *17* 484—537.

BROWN, G. L., Release of sympathetic transmitter by nerve stimulation. In Adrenergic mechanisms. Churchill, London 1960.

BUCHTHAL, F. and J. LINDHARD, Transmission of impulses from nerve to muscle fibre. *Acta physiol. scand.* 1942. *4* 136—148.

CHANG, C. C., Studies on the mechanism of curare-like action of Bungarus multicinctus venom. I. *J. Formosan med. Ass.* 1959 a. *58* 315—323.

CHANG, C. C., Studies on the mechanism of curare-like action of Bungarus multicinctus venom. II. *J. Formosan med. Ass.* 1959 b. *58* 416—426.

CHOU, T. C., A method of estimating curare-like activity on the isolated phrenic nerve diaphragm preparation of the rat. Brit. J. Pharmacol. 1947 2. 1—7.

CURTIS, D. C. and J. C. ECCLES, Synaptic action during and after repetitive stimulation. *J. Physiol.* (Lond.) 1960. *150* 357—398.

DALE, H. H., W. FELDBERG and M. VOGT, Release of acetylcholine at voluntary motor nerve endings. *J. Physiol.* (Lond.) 1936. *86*. 353—380.

DETTBARN, W. D., Effect of curare on conduction in myelinated isolated nerve fibres of the frog. *Nature* (Lond.) 1960. *186*. 891—892.

DOUGLAS, W. W. and J. M. RITCHIE, The excitatory action of acetylcholine on cutaneous non-myelinated fibres. *J. Physiol.* (Lond.) 1960. *150*. 501—514.

ECCLES, J. C., B. KATZ and S. KUFFLER, Nature of the "endplate potential" in curarized muscle. *J. Neurophysiol.* 1941 *4* 363—387.

ECCLES, J. C., D. PIHL and A. W. LILEY, Factors controlling liberation of acetylcholine at neuromuscular junction. *Amer. J. phys. Med.* 1959 *38*. 96—105.

ECCLES, R., Intracellular potentials recorded from a mammalian sympathetic ganglion. *J. Physiol.* (Lond.) 1955. *130*. 572—584.

ENGBAEK, L., Investigations on the course and localisation of magnesium anesthesia. *Acta pharmacol.* (Kbh.) 1948. Suppl. 4.

FLEISCH, A., I. SIBUL and M. KAELIN, De l'apparition de l acetylcholine dans le sang. *Arch. int. Physiol.* 1936. *44* 24—36.

GREEN, A. F. The effect of bretylium and allied agents on the adrenergic neurones. In Adrenergic mechanisms. Churchill, London. 1960.

GRUNDFEST, H. and D. NACHMANSOHN, Mode of blocking of axonal activity by curare and inhibition of acetylcholinesterase. *Nature* (Lond.) 1952. *169*. 190.

HOFMANN, F. B., Studien über Tetanus. *Pflügers Arch. ges. Physiol.* 1903. *95*. 484—532.

HUTTER, O. F., Post-tetanic restoration of neuromuscular transmission blocked by d-tubocurarine. *J. Physiol.* (Lond.) 1952. *118*. 216—227.

KOKETSU, K., Action of tetraethylammonium chlorid on neuromuscular transmission in frog. *Amer. J. Physiol.* 1958. *193*. 213—218.

KRNJEVIĆ, K. and R. MILEDI, Failure of neuromuscular propagation in rats. *J. Physiol.* (Lond.) 1958. *140*. 440—461.

LAPORTE, Y. and R. LORENTE DE NÓ, Potential changes evoked in a curarized sympathetic ganglion by presynaptic volleys of impulses. *J. cell. comp. Physiol.* 1950. *35*. Suppl. 2. 61—105.

LILEY, A. W. An investigation of the spontaneous activity at the neuromuscular junction of the rat. *J. Physiol.* (Lond.) 1956. *132*. 650—666.

LILEY, A. W. and K. A. K. NORTH, An electrical investigation of effects of repetitive stimulation on mammalian neuromuscular junction. *J. Neurophysiol.* 1953. *16*. 507—527.

LILLEHEIL, G. and K. NAESS, Presynaptic effect of d-tubocurarine. *Experientia* (Basel) 1960. *16*. 550—551.

LILLEHEIL, G. and K. NAESS, Notes on the effect of increased K Cl-concentration on the neuromuscular transmission. *Acta physiol. scand.* 1961.

MASLAND, R. L. and R. S. WIGTON, Nerve activity accompanying fasciculation produced by prostigmin. *J. Neurophysiol.* 1940. *3*. 269—275.

NACHMANSOHN, D. Chemical and molecular basis of nerve activity. *Academic Press.* New York and London. 1959.

NAESS, K., Effects of brief and protracted curarization. *Acta pharmacol.* (Kbh.) 1952. *8*. 400—408.

NAESS, K., Addendum to effects of brief and protracted curarization. *Acta pharmacol.* (Kbh.) 1953. *9*. 196—197.

NAESS, K. and A. STORM-MATHISEN, Fatigue of sustained tetanic contractions. *Acta physiol. scand.* 1955. *34*. 351—366.

OTSUKA, M. and M. ENDO, Presynaptic nature of neuromuscular depression in the frog. *Nature* (Lond.) 1960. *188*. 501—502.

RIKER, W. F. JR., J. ROBERTS, F. G. STANDAERT and H. FUJIMORI, The motor nerve terminal as the primary focus for drug-induced facilitation of neuromuscular transmission. *J. Pharmacol.* 1957. *121*. 286—312.

RIKER, W. F. JR., G. WERNER, J. ROBERTS and A. KUPERMAN, The presynaptic element in neuromuscular transmission. *Ann. N. Y. Acad. Sci.* 1959. *81*. 328—344.

RIKER, W. K. and Z. SZRENIAWSKI, The pharmacological reactivity of presynaptic nerve terminals in a sympathetic ganglion. *J. Pharmacol.* 1959. *126*. 233—238.

ROSENBLUETH, A. and R. S. MORISON, Curarization, fatigue and Wedensky inhibition. *Amer. J. Physiol.* 1937. *119*. 236—256.

STOVNER, J. The anticurare activity of tetraethylammonium (TEA). *Acta pharmacol.* (Kbh.) 1958 a. *14*. 317—332.

STOVNER, J. The facilitation of neuromuscular transmission induced by tetraethylammonium. *Oslo Univ. Press.* Oslo 1958 b.

STRAUGHAN, D. W. The release of acetylcholine from mammalian motor nerve endings. Brit. J. Pharmacol. 1960. *15*. 417—424.

THESLEFF, S., Motor end-plate desensitization by repetitive nerve stimuli. *J. Physiol.* (Lond.) 1959. *148*. 659—664.

WEDENSKY, N. E., Die Erregung, Hemmung und Narkose. *Pflüg. Arch. ges. Physiol.* 1903. *100*. 1—144.

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Effect of Reserpine on the Release of Catecholamines from Isolated Nerve and Chromaffin Cell Granules

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Abstract

Euler, U S. and F Lishajko. *Effect of reserpine on the release of catecholamines from isolated nerve and chromaffin cell granules.* Acta physiol. scand. 1961 52 137—145 — In suspension of bovine splenic nerve granules, reserpine in concentrations of $10^{-4}-10^{-8}$ M strongly inhibits the spontaneous release of noradrenaline. The catecholamine releasing ac tion of tyramine on nerve granules is unaffected by low concentrations of reserpine, but is almost completely blocked by concentrations of 10^{-4} M. Reserpine is less active as inhibitor on suprarenal medullary granules from the rabbit and still less on cats medullary granules. The spontaneous release occurs at lower rate in rabbit's medullary granules than in those from the cat. N difference in the rate of release was found between adrenaline and noradrenaline from the cat me dullary granules. After administration of dopa 100 mg/kg i m the rabbit the rate of release from the medullary granules was greatly enhanced. The possible action of reserpine as enzyme inhibitor is briefly discussed.

In a previous paper by Euler nd Lishajko (1960 b) t was reported that noradrenaline was released at an increased rate from isolated and resuspended bovine splenic nerve granules on incubation with reserpine. The concentrations necessary to cause a marked release were high, however thus about 0.5 mM (0.3 mg/ml) reserpin (as phosphate) per ml of suspension fluid was required to cause about 90 per cent release as compared with 30—40 per cent spontaneous" release during 30 min t + 20° C. In concentrations below 0 1 mM no releasing effect was observed. Similar effects were obtained with granules from the heart of two cyclostomes, *Myxine glutinosa* and *Petromyzon fluviatilis* (Östlund *et al.* 1960)

Subsequent experiments with a slightly modified technique have revealed an effect of reserpine of a different kind. It was thus observed that incubation of catecholamine granules from nerves with reserpine in low concentrations to a large extent prevented the release of noradrenaline. In the present report are described some results obtained with granules from nerves and suprarenal chromaffin cells on incubation with reserpine.

Method

Catecholamine containing granules were prepared from bovine splenic nerves by the method described previously (EULER and LISHAJKO 1961 a) based on squeezing the carefully dissected nerve trunks between nylon cylinders in the cold and uptake of the press juice in 0 13 m K phosphate at pH 6—7

While in the previous study the sediment was resuspended in K-phosphate the suspension was used directly in the present experiments, after removal of larger particles by centrifugation t 1 000 g for 5 min at 0—4 C. Almost no spontaneous release of noradrenaline occurred during 3 hours when the suspension was kept t a temperature of + 4 C at pH 6—7 Lyophilized reserpine phosphate (kindly supplied by Dr A. Plummer Ciba, Summit, N J) was added in aqueous solution To the controls was added the same quantity of redistilled water

After addition of reserpine the suspension was incubated for 2 hours in water bath at + 20° C. One sample was kept t + 4 C to serve as an absolute control. One or two aliquots of the original suspension were incubated without reserpine in order to measure the spontaneous release.

In a number of experiments tyramine hydrochloride was added to the incubation fluid.

At the end of the incubation period the samples were centrifuged for 30 min at 50,000 × g at + 4 C and the sediment extracted with hydrochloric acid and metaphosphoric acid added according to the method of EULER and LISHAJKO (1961) After ...on and removal of the sediment the noradrenaline was estimated fluori...y according to the method of EULER and LISHAJKO (1961 b)

In special tests it was established that reserpine does not interfere with the fluorimetric estimation of noradrenaline in amounts up to 250 μg which are considerably higher than those occurring in the samples of the present study Similar controls were made with tyramine

Granules were also prepared from the suprarenal glands of the rabbit and the cat in the same way as described above

Results

1 Splenic nerve granules

a. Reserpine.

Fig 1 shows the effect of incubation of splenic nerve granules with different concentrations of reserpine. As seen in the figure reserpine causes a very pronounced inhibition of the release in concentrations of 1 to 100 μg per ml. Even in a concentration as low as 0.1 μg/ml a marked inhibition of the release is observed. By pretreatment for 30 min at + 4 C the inhibitory action of reserpine is observed even n a concentration of 0 01 μg per ml or 1.6 × 10⁻ M

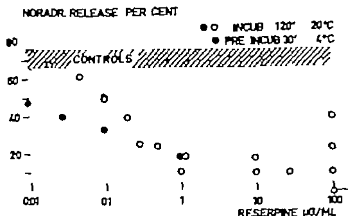


Fig 1 Inhibitory effect of reserpine on the release of noradrenaline from suspension of isolated bovine splenic nerve granules. Controls, spontaneous release. Empty circles per cent NA release after 2 hours incubation at + 20° C, pH 6—7 Filled circles Same, with pre-incubation for 30 min at + 4° C.

No difference in the degree of inhibition has been noticed when the pH was varied between 6 and 7 in the incubated suspension, if reserpine was present in concentrations of 10 µg/ml or lower In higher concentrations pH 6 was used in order to prevent precipitation of reserpine.

b. *Reserpine and tyramine.*

It has been shown previously that tyramine can cause a release of noradrenaline from isolated granules (SCHÜMANN 1960 EULER and LISHAJKO 1960 a) It therefore seemed of interest to study whether the releasing effect of tyramine was influenced by reserpine in concentrations which inhibit the release. As standard concentration of tyramine 10 µg/ml (7.3×10^{-5} M) has been chosen since this consistently has shown good releasing effect.

Fig 2 shows the time course of the effect of tyramine alone on the release of noradrenaline from a suspension of splenic nerve granules in comparison with controls. Since it was shown that tyramine is partly inactivated by monoamineoxidase present in a resuspension of the sediment (W Hardegg, personal communication) a second dose of tyramine was added after 1 hour in later experiments. The inactivation of tyramine is possibly reflected in a slight deviation of the release curve as seen in Fig 2.

Fig. 3 demonstrates that the increased release of noradrenaline caused by tyramine is abolished in the presence of reserpine in a concentration of 10 µg/ml. In very low concentrations, however 0.01—0.03 µg per ml, reserpine, though still inhibiting the "spontaneous release, does not block the action of tyramine In the intermediary range of concentrations, 0.1—1 µg/ml, reserpine exerts a gradually increasing blocking effect on the tyramine action. In these

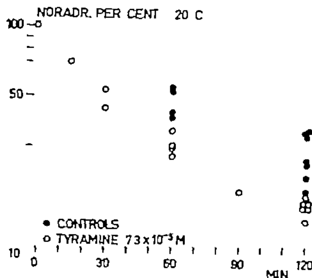


Fig 2. Release of noradrenaline from suspension of bovine splenic nerve granules at pH 7.0 during incubation at + 20 C. Spontaneous release and effect of tyramine 7.3×10^{-5} M (10 µg per ml). Ordinate: per cent noradrenaline in sediment after incubation.

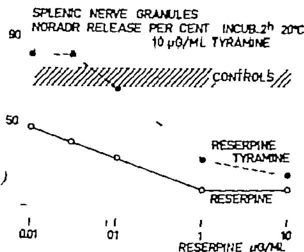


Fig 3. Effect of reserpine on the noradrenaline releasing action of tyramine on isolated bovine splenic nerve granules. Incubation for 30 min at + 4 C followed by 2 hours at + 20 C. Effect of tyramine alone and control release at + 20 C for 2 hours indicated. Tyramine 7.3 10^{-5} M in all experiments, reserpine concentration on the abscissa.

experiments the granule suspension was pre-incubated with reserpine for 30 min at + 4 C which did not influence the noradrenaline content of the granules. The controls were treated in the same way. From these experiments it is evident that reserpine in concentrations of the same order as those of tyramine almost completely inhibits the effect of tyramine. This appears of interest since tyramine is assumed to act by substituting noradrenaline in the granules on account of its stronger basic properties (SCHÜMANN personal communication).

Table I. Release of adrenaline (A) and noradrenaline (NA) after incubation of cat suprarenal medullary granules for 2 h at + 20° C, pH 7.0 with reserpine and tyramine in per cent of amount present in the suspension incubated 2 h at + 4° C. Proportion A : NA in sediment of original suspension, 1 : 2.5 and 1:0.97 respectively Expt. I and II

	A		NA	
	I	II	I	II
Control	75	89	77	89
Reserpine 1.6 × 10^{-4} M	69	79	75	86
Tyramine 7.3 × 10^{-4} M	85	94	86	95
Reserpine + tyramine	78	92	84	94

II. Suprarenal medullary granules

A series of experiments were also made with granules from the suprarenal medulla of the cat and the rabbit.

a. *Cat.*

The suprarenals were removed under nembutal anesthesia and granules prepared. The suspension of granules was incubated with reserpine and tyramine as in previous experiments. Table I shows the effect of incubation on the release of adrenaline and noradrenalin from the granules.

As seen in the table reserpine has a very slight inhibitory effect on the "spontaneous" release as compared with the action on splenic nerve granules. Tyramine has a moderate releasing effect on noradrenaline as well as adrenaline, which is in keeping with the results reported by Schümann (1960) for bovine medullary granules. Even in the presence of reserpine 1.6 × 10^{-4} M, tyramine has almost the same releasing effect as without reserpine, in marked contrast to the effects observed with nerve granules (Fig. 4).

b. *Rabbit.*

The rabbits were killed by a blow on the neck and medullary granules prepared in a similar way as for the cat. The inhibitory effect of reserpine was somewhat more marked than on the cat' medullary granules. In Table II are given the results of incubation experiments of the same kind as in Table I with granules from untreated animals and after administration of dopa. Dopa was given i.v. in a dose of 100 mg per kg after dissolution in Serpasil® solvent (1 ml for 100 mg) and dilution in 15—20 ml saline under warming to about 40° C. Figures are only given for adrenaline since the relative amounts of noradrenaline were small.

In spite of the fairly large variations between the single experiments it can be seen from Table II that reserpine exerts an inhibitory action on the release of adrenaline in 5 of the 6 experiments. Tyramine increased the release likewise

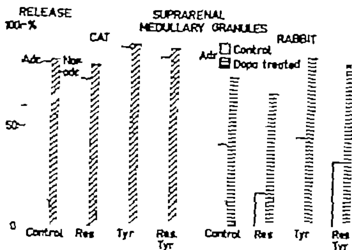


Fig. 4. Release of adrenaline and noradrenaline (cat, left) and adrenaline (rabbit, right) after incubation of medullary granules for 2 hours at + 20° C, pH 7.0 with reserpine and tyramine, in per cent of amount present in a suspension incubated 2 hours at + 4° C. Bars with horizontal stripes (right) indicate actions on medullary granules after pretreatment of rabbit with dopa 100 mg/kg

Table II. Release of adrenaline after incubation of rabbit suprarenal medullary granules for 2^h at + 20° C, pH 7.0 with reserpine and tyramine in per cent of the amount present in a suspension incubated 2^h at + 4° C. Expt. I—VI

	Untreated I	II	III	Average	1^h after 100 mg/kg dopa L IV	V	VI	Average
Control	26	37	60	41	74	65	87	75
Reserpine 1.6 10^{-4} M	11	22	17	17	74	54	74	67
Tyramine 7.3 10^{-4} M	37	45	54	45	83	81	91	85
Reserpine + tyramine	33	49	18	33	76	82	87	82

in 5 of 6 experiments. On the other hand reserpine was relatively inefficient in preventing the releasing effect of tyramine (Fig. 4). Using a high concentration, 200 µg/ml of reserpine, a releasing effect was noted, however being about twice as strong as the spontaneous release at + 20° C.

Discussion

While the "spontaneous" release of noradrenaline from bovine splenic nerve granules did not differ markedly from that in cat's medullary granules, both were considerable higher than the release from rabbit's medullary granules.

These behaved in this respect more like the bovine medullary granules (HILLARP and NILSON 1954)

With regard to the effect of reserpine on the release of catecholamines from storage granules of various origins the picture is complicated. In resuspended granules from bovine splenic nerves it was shown previously (EULER and LISHAJKO 1960 b) that reserpine increased the rate of release in concentrations of about 0 1 mg/ml and higher This finding has been confirmed for granules both from nerves and from chromaffin cells in later experiments, but it was also found that when reserpine was added in lower concentrations directly to the suspension of splenic nerve granules and the mixture incubated at + 20° C for various times t had an opposite effect which was very marked. Thus an almost complete inhibition of the release was observed with reserpine in concentrations down to 1 μg/ml or 1.6 × 10⁻⁶ M. A definite inhibitory effect was noticed even in a concentration of 1.6 × 10⁻⁸ M if the granule suspension was preincubated with reserpine at low temperature.

This oligodynamic effect of reserpine also modifies the releasing action of tyramine. While t will not prevent the tyramine action in concentrations of about 5 × 10⁻⁸ M, its inhibitory action of the tyramine releasing effect increases with higher concentrations. Thus the tyramine releasing action is almost completely blocked when reserpine is present in a concentration of 1 6 × 10⁻⁶ M. This action is difficult to explain on the basis of the assumption that tyramine simply substitutes noradrenaline in the granules on account of its stronger basicity No explanation can at present be given for the striking effect of reserpine in low concentrations on the noradrenaline release on incubation of splenic nerve granules, but t appears not unplausible that reserpine selectively blocks some enzymatic system involved in the release. Since reserpine is known to cause a depletion of the catecholamine stores in nerves and organs it may be assumed that it also blocks the mechanism by which storage is effected (CARLSSON et al. 1957 BERTLER 1961) A similar effect has been postulated for bretylium and guanethidine by ZAIMIS (1960) The depletion of organs may be explained by a continuous leakage of the transmitter from the stores, not followed by repletion. Recently MUSCHOLL (1960) has shown that infused noradrenaline is not taken up by the hearts of reserpine treated rats. It is also interesting to note that SANO et al. (1960) found a blocking effect of reserpine on the uptake of 5-HT or adrenaline by thrombocytes.

In this context it should be recalled that ABOOD and ROMANCHEK (1956—57) observed a marked inhibition of oxidative phosphorylation in brain mitochondria by reserpine in a concentration of 2 × 10⁻⁶ M.

The inhibitory effect of reserpine on the release of catecholamines is less marked on suprarenal medullary granules of the rabbit and very slight on such granules from the cat. STJÄRNE and SCHAPIRO (1959) observed no effect of reserpine in concentrations of 0.05—5 μg/ml on isolated bovine med llary

granules. Using 200 μg/ml we have observed a marked releasing effect on the rabbit medullary granules, however. This effect is in keeping with the direct releasing action of reserpine when given intravenously in large amounts (MUSCHOLL and VOGT 1957). Similarly the blocking effect of reserpine on the releasing effect of tyramine is weaker than in the case of splenic nerve granules. This is particularly obvious after pretreatment of the rabbit with dopa which greatly enhances the spontaneous release of adrenaline from isolated medullary granules on incubation at + 20° C. It is also worth mentioning that no difference was observed between the release from noradrenaline and adrenaline storing granules in the cat's medulla.

The quantitative rather than qualitative difference in action of reserpine on the catecholamine storage granules from different tissues may be correlated with the differences reported between the depleting effect of reserpine on different organs and in different species. Thus the cat's suprarenal medulla is known to respond less readily to reserpine.

The reason why resuspended splenic nerve granules should tend to react with increased rate of release for reserpine in concentrations which have an inhibitory effect on the original suspension is not clear but may be due to the loss of some factor or factors in the resuspended preparation. Even in concentrations of 0.2 mg/ml, reserpine did not enhance the release of noradrenaline when added directly to the original suspension of nerve granules.

The strong enhancement of the spontaneous release in rabbit's medullary granules after pretreatment of the animal with dopa seems to indicate an important disturbance in the granules. It appears less probable that this effect is due to the formation of dopamine since this does not influence the release (SCHÜMANN 1960, EULER and LISHAJKO 1960 a) from granules either from nerves or from medullary cells.

This work was supported by grants from the Swedish Medical Research Council and the Air Force Office of Scientific Research of the Air Research and Development Command, United States Air Force, through its European Office, which is gratefully acknowledged.

References

ABOOD, L. G. and L. ROMANCHEK, The chemical constitution and biochemical effects of psychotherapeutic and structurally related agents. *Ann. N. Y. Acad. Sc.* 1956—57. *66.* 812—825.

BERTLER, Å., Effect of reserpine on the storage of catechol amines in brain and other tissues. *Acta physiol. scand.* 1961. *51.* 75—83.

CARLSSON, A., E. ROSENGREN, Å. BERTLER and J. NILSSON, Effect of reserpine on the metabolism of catechol amines. *Psychotropic Drugs* 1957. 363—372.

EULER, U. S. v. and F. LISHAJKO, Release of noradrenaline from adrenergic transmitter granules by tyramine. *Experientia (Basel)* 1960 a. *16.* 376—377.

EULER, U. S. v. and F. LISHAJKO, Effect of reserpine on the release of noradrenaline from transmitter granules in adrenergic nerves. *Science* 1960 b. *132.* 351—352.

EULER, U. S. v. and F. LISHAJKO, Noradrenaline release from isolated nerve granules. *Acta physiol. scand.* 1961 a. *51* 193—203.

EULER, U. S. v. and F. LISHAJKO, Improved technique for the fluorimetric estimation of catecholamines. *Acta physiol. scand.* 1961 b. *51* 348—356.

HILLARP, N.-Å. and B. NILSON, The structure of the adrenaline and noradrenaline containing granules in the adrenal medullary cells with reference to the storage and release of the sympathomimetic amines. *Acta physiol. scand.* 1954. *31* Suppl. 113. 79—107

MUSCHOLL, E., Die Hemmung der Noradrenalin-Aufnahme des Herzens durch Reserpin und die Wirkung von Tyramin. *Arch. exp. Path. Pharmak.* 1960. *240* 234—241

MUSCHOLL, E. and M. VOGT. The concentration of adrenaline in the plasma of rabbits treated with reserpine. *Brit. J. Pharmacol.* 1957 *12* 532—535.

ÖSTLUND, E., G. BLOOM, J. ADAMS-RAY, M. RITZÉN, M. SIEGMAN, H. NORDENSTAM, F. LISHAJKO and U. S. v. EULER, Storage and release of catecholamines, and the occurrence of a specific submicroscopic granulation in hearts of cyclostomes. *Nature* (Lond.) 1960. *188.* 324—325.

SANO, I. Y. KAKIMOTO, K. TANIGUCHI and M. TAKESADA, Aktiver Transport der aromatischen Monoamine. *Klin. Wschr.* 1960. *38.* 41—45.

SCHUMANN, H. J. Ueber die Freisetzung von Brenzcatechinaminen durch Tyramin. *Arch. exp. Path. Pharmak.* 1960. *238.* 41

STJÄRNE, L. and S. SCHAPIRO, Effect of reserpine on secretion from the denervated adrenal medulla. *Nature* (Lond.) 1959. *184* 2023—2024.

ZAIMIS, E., In Adrenergic Mechanisms. *J. & A. Churchill*, London 1960. pp. 171.

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From the Department of Physiology Karolinska Institutet, Stockholm, Sweden

The Effect of Choline 2,6-Dichloro Phenyl Ether Bromide (TM 25) on Organ Content and Urinary Excretion of Catecholamines

By

S. SCHAPIRO[1]

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Abstract

SCHAPIRO, S. *The effect of choline 2,6-dichloro phenyl ether bromide (TM 25) on organ content and urinary excretion of catecholamines.* Acta physiol scand. 1961 52 146—149. — Daily injections of choline 2,6-dichloro phenyl ether bromide (TM 25) in the rat greatly reduces the urinary output of noradrenaline and to lesser extent that of adrenaline. After 5—6 days of treatment with TM 25 the catecholamine content of adrenals, heart and spleen is considerably reduced. It is inferred from the results that TM 25 interferes with the production, release or storage of catechol amines in the adrenergic nerves and possibly in the adrenals.

Several contributions have been made during recent years towards elucidating the action pattern of the choline phenyl ethers. As a result of these studies it has been demonstrated that some compounds in this group such as TM-6 (choline-p-tolyl ether bromide) and TM 10 (choline 2,6-xylyl ether bromide) act as inhibitors of amine oxidase in vitro (BROWN and HEY 1952 1956 GRIESEMER and WELLS 1956) though not in vivo (CORNE and GRAHAM 1957). The catecholamine content of organs does not seem to have been studied after administration of TM-6 but it has been reported that it does not alter the catecholamine excretion in urine in the cat (CORNE and GRAHAM 1957). TM 10 on the other hand, blocks adrenergic nerve action (EXLEY 1956 1957 BAIN and FIELDEN 1956). It has a slight depleting effect on the content of catecholamines in the suprarenals of rats when administered daily over 2 weeks (COUPLAND and EXLEY 1957). The urinary excretion of catecholamines as a convenient

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Table I. Effect of intraperitoneal injection of 4 mg/kg TM-25 on organ catechols (μg/g) in rats

	Controls 10 animals		Group A 6 days TM 25 5 animals		Group B 5 days TM-25 (after 7 daily inj. of saline) 5 animals		Group C One week after end of treatment with TM-25 5 animals	
	NA	A	NA	A	NA	A	NA	A
Spleen	0.96	—	0.21	—	0.13	—	0.42	—
Heart	0.62	—	0.43	—	—	—	0.46	—
Adrenals	280	900	79.5	560	56	300	140	864

relative measure of the rate of release, does not seem to have been investigated. No depletion of the organ content of catecholamines was observed in short term experiments on guinea-pigs (EULER and LISHAJKO, personal communication).

In view of the marked effects induced by small changes in the composition of these compounds it seemed of interest to study the organ content and urinary excretion of catecholamines after administration of TM 25 the dichloro analogue of TM 10 which was kindly placed at our disposal by Dr W. A. BAIN

Methods

Five female rats (weight 222—310 g) were placed in community cage and TM 25 was injected daily for 6 days intraperitoneally into each animal in a dose of 4 mg per kg in 0.5 ml saline. After this period the rats were sacrificed by blow on the head, and adrenals, spleens and hearts analyzed for catechols by the fluorimetric technique of EULER and FLODING (1955) after mincing and extraction with 5 per cent trichloroacetic acid (Group A).

In another series 10 female rats were placed in community metabolism cage and injected with 0.5 ml saline daily i. p. for 7 days. Urine was collected daily during this control period. TM 25 was then injected daily in the same dose as in group A for 5 days. Five rats were then sacrificed as previously and organs analyzed for catechols (Group B) The remaining 5 rats received saline for an additional 7 day period and were then sacrificed as before (Group C) Urine was saved over the first 5 days and analyzed for catechols Adrenals, hearts and spleens were also analyzed for catechols as described above. Control values for normal organ catechols were obtained from 10 normal rats which were sacrificed by blow on the head.

Results

1 *Effect of TM 25 on urinary catechols* In Fig 1 is shown the catechol excretion values in ng/kg/hr before, during and after treatment with TM-25. As seen in the figure the drug drastically reduced the output of both adrenaline and noradrenaline. Following cessation of treatment the adrenaline remained low for several days, while noradrenaline tended to return to control levels.

2. *Effect of TM 25 on organ catechols* After treatment for 5 and 6 days with

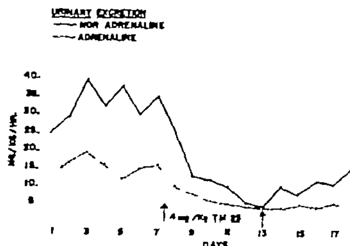


Fig. 1. Urinary catechols before (10 animals) during (10 animals) and after (5 animals) daily administration of TM 25 for 7 days

TM 25 the catechol content of the spleen and adrenals was reduced to approximately 1/5—1/2 of the resting level (Table I). The heart apparently was more resistant to the depleting effects. After one week without treatment, organ catechols were returning to control levels, although the normal figures were not quite reached.

Discussion

The results reported here have shown that TM 25, like TM 10 (COUPLAND and EXLEY 1957) diminishes the catechol content of the adrenals in the rat. We have further observed that the spleen loses most of its catecholamines when these were examined at the end of 5—6 days treatment with TM 25. The fall in catecholamines in the heart was less marked, however. Simultaneously with these organ changes, the urinary excretion of noradrenaline and adrenaline is decreased. As the organ contents are partially restored to normal levels 7 days after cessation of treatment, the urinary catechols also tend to return to normal values.

It has been recently shown (SCHAPIRO 1958) that a catecholamine blocking agent, dibenzyline, in a dose of 10 mg/kg causes an increased urinary excretion of noradrenaline in rats and depleted various organs of their catechols. BENFEY, MAZURKIEWICZ and MELVILLE (1958) also showed that sympathetic blocking drugs increased the fraction recovered in urine after injection of adrenaline and noradrenaline. It is indeed possible that these blocking agents compete for metabolic receptor sites (as contrasted with effector receptor sites) with a resultant "overflow" of catechols into the blood and subsequently into the urine, in a way analogous to that described in its first step by BROWN and GILLESPIE (1957).

The present results are obviously in contrast to the effect of the sympatholytic agents, although TM 25 has been shown to have a short-lasting adrenolytic action (McLean *et al.* 1960) If the TM compounds acted only as inhibitors of amine oxidase and blocked liver inactivation of adrenaline (Griesemer and Wells 1956) one would expect an increased urinary excretion of catechols The decreased output actually found agrees with the suggestion of Coupland and Exley that in vivo an inhibition of catechol synthesis may actually occur in addition to the blocking of the release. This action seems to be on the adrenals as well as on the adrenergic nerves although the more marked fall in the noradrenaline excretion in urine suggests that the greater part of the effect is on the adrenergic nerves. This is further supported by the demonstration that TM 10 prevents the transformation of dopamine to noradrenaline in human chromaffin tissue (Bain and Fielden 1957)

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References

Bain W. A. and R. Fielden Preliminary experiments on the mode of action of choline 2 6-xylyl ether bromide on adrenergic nerves. *J Physiol.* (Lond.) 1956. *133.* 70 P—71 P

Bain W. A. and R. Fielden, In-vitro formation of noradrenaline from dopamine by human tissue. *Lancet* 1957 Sept. 7 472—473.

Brody B. G., I. Marcharkiewicz and K. I. Melville, Changes in epinephrine and norepinephrine excretion in urine following injection of sympathetic blocking agents. *J Pharmacol.* 1958. *122.* 5 A—6 A.

Brown, B. G. and P Hey Substituted choline aryl ethers as inhibitors of amine oxidase. *J Physiol.* (Lond.) 1952. *118.* 15 P

Brown B. G. and P Hey Choline phenyl ethers as inhibitors of amine oxidase. *Brit. J Pharmacol.* 1956. *11* 58—65.

Brown, G L. and J S. Gillespie, The output of sympathetic transmitter from the spleen of the cat. *J Physiol.* (Lond.) 1957 *138.* 81—102.

Corne, S. J and J D P Graham, The effect of inhibition of amine oxidase in vivo on administered adrenaline, noradrenaline, tyramine and serotonin. *J Physiol.* (Lond.) 1957 *135.* 339—349

Coupland, R. E. and K. A. Exley Effects of choline 2 6-xylyl ether bromide upon the suprarenal medulla of the rat. *Brit. J Pharmacol.* 1957 *12.* 306—311

Euler, U S. and I. Floding, Fluorimetric estimation of noradrenaline and adrenaline in urine. *Acta physiol. scand.* 1955. *33.* Suppl. 118. 57—62.

Exley K. A., The blocking action of choline 2 6-xylyl ether bromide on adrenergic nerves. *J Physiol.* (Lond.) 1956. *133.* 70 P

Exley K. A., The blocking action of choline 2 6-xylyl ether bromide on adrenergic nerves. *Brit. J Pharmacol.* 1957 *12.* 297—305.

Griesemer, E. C. and J A. Wells, Abolition of epinephrine inactivating properties of liver by inhibitors of monoamine oxidase. *J Pharmacol.* 1956. *116.* 282—286.

McLean R. A., R. J Geus, R. J. Mohrbacher, P A. Mattis and G. E. Ullyot A series of 2,6-disubstituted phenoxyethyl ammonium bromides with true sympatholytic properties. *J Pharmacol.* 1960. *129.* 11—16.

Schapiro, S., Effect of catechol amine blocking agent (dibenzyline) on organ content and urine excretion of noradrenaline and adrenaline. *Acta physiol. scand.* 1958. *42.* 371—375.

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Taste Functions in the Carp

An Electrophysiological Study on Gustatory Fibres

By

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Abstract

J. Konishi and Y. Zotterman. *Taste functions in the carp. An electrophysiological study on gustatory fibres.* Acta physiol. scand. 1961. 52. 150—161. — The electrical responses of the taste fibres in carp (*Cyprinus carpio* (L.)) were recorded during the application of various sapid substances. A comparison of the integrated electrical responses from the glossopharyngeal nerve innervating the palatal organ, the facial nerve innervating the lining of the mouth and the barbels, and the branchial nerve innervating the gill rakers showed that the palatal organ seems to be the principal taste organ of this fish. The records from the glossopharyngeal nerve revealed that this nerve contains chemoceptive fibres responding to a great variety of sapid solutions. The response to acid and to sucrose was particularly strong, while the integrated response to 0.5 M NaCl always was considerably weaker. The application of water to the palatal organ had no stimulating effect. Especially noteworthy was a generally quite strong response to the application of human saliva.

A study of the records from 114 single taste fibres from the glossopharyngeal nerve revealed that the fibres could be divided into seven groups according to their response pattern. Highly specific response to NaCl was encountered in 14 fibres. Acetic acid (0.005 M) seemed to stimulate all taste fibres, except salt fibres. Specific response to weak acid was noticed in 3 fibres. The fibres which responded to human saliva were also stimulated by sucrose, except for 3 fibres. Touching the surface of the palatal organ did not stimulate any taste fibres.

In fishes receptor structures resembling taste buds in mammals have been described by many anatomists. These receptors are situated around the mouth, in the oral cavity and on the gill rakers, as well as in barbels of various fishes. They are found particularly densely in the roof of the mouth of the carp, in its

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palatal organ. This organ is supplied by nerve fibres from the glossopharyngeal nerve. HOAGLAND (1933) first recorded electrical responses from the facial nerve of the catfish in response to various sapid solutions applied on the barbels. In this research we have tried to obtain more detailed data about the gustatory mechanisms of a fresh water fish.

Methods

A total of 78 carps (*Cyprinus carpio* (L.)) with n average body length of 28 cm were used in this study. The fish was decapitated and the vagal lobe as well as the medulla oblongata were destroyed. Integrated records in response to sapid solutions were obtained from bundles of the glossopharyngeal nerve, from the facial nerves innervating the linings of the mouth, and from the nerve bundles innervating the gill rakers. In order to expose the bundles of the glossopharyngeal nerve, which innervate the palatal organ, this organ was carefully separated from the palatal cartilage and lifted by hooks. In all experiments we used the bundle innervating the anterio-lateral part of the palatal organ, running closely to the anterior margin of the palatal cartilage because this bundle offered the longest stretch of nerve (available length for recording is bout 5 mm) The cartilage was removed in order to facilitate further dissection. The nerve was lifted on to a platinum electrod attached to a micromanipulator and connected to the input of an RC coupled amplifier The other input was attached to the tissues and grounded. Responses of the entire nerv were recorded, using an electronic integrating device which has been described in earlier papers.

To make single fibre preparations, the nerve was placed on small platform with black surface offering good contrast to the nerve strands. The epineural sheath was then dissected off and the nerve split into small bundles, from which fine strands were separated by further dissection under binocular microscope. Under Ringer' solution, the entire glossopharyngeal nerve preparations could be kept responsive for four hours or more The facial nerve was exposed from the orbit. The recording from the branchial nerv was done in isolated preparation.

The test solutions were pplied by a specially made apparatus incorporating signalling device. The taste solutions mainly tested were sodium chloride (0.5 M) sucrose (0.5 M) quinine hydrochloride (0.01 M) and acetic acid (0.003 M, pH 3.8) in queous solutions. Some fibres were also tested with solutions of saccharine, glycerol (0.5 M) and ethylene glycol (0.5 M)

Results

Integrated responses

Glossopharyngeal nerve

Water hardly stimulates any of the chemoreceptors of this fish, and especially after a previous rinse with water The palatal nerve responded to all fou conventional classes of taste substances salt, sucrose quinine and acid especially strongly to acid and sucrose (Fig. 1) In all the nerve preparations tested, glycol and glycerol also elicited a positive response, while saccharine gave a very feeble response if any The responses to repeated application of a test solution decreased, even if nterspersed with water rinses. It is particularly notable that human saliva had a strong effect on the chemoceptors, and gave a much bigger integrated response than the 0.5 M NaCl and 0.01 M quinine sol tions. The

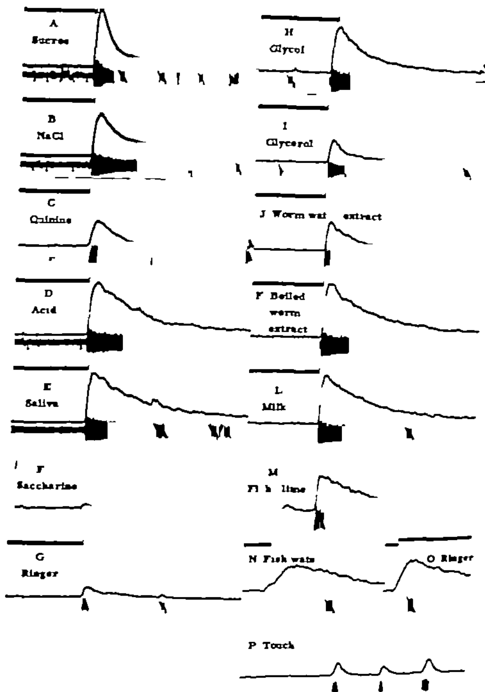


Fig 1 Electrical responses from the entire glossopharyngeal nerve of carp to the application of various solutions to the palatal organ and to touch. In each tracing are recorded, from top to bottom the signal showing the moment of application, the integrated response and the direct spike response. Records N and O were recorded by high amplification. Time in sec

response to fish Ringer's solution was quite small, even after a previous rinse with water Water extract of earth worm also produced a positive response, and a boiled extract was particularly effective. Milk (1 / dry milk in distilled water) also produced a massive response. The mucous substances covering the body surface of the fish gave a positive response. It is a surprising fact that the chemoceptors responded, though in small degree, even to the water in which fish had been kept (Fig 1 N) The response to dilute solutions as well as to the water the fish had been kept in was usually found to rise rather slowly to at tain a slowly declining plateau. It is interesting to note that the responses to sugar were comparatively phasic in both integrated and direct spike responses, in spite of large magnitude, while those to saliva as well as to worm extract and milk lasted much longer

Besides chemoceptive and motor fibres the glossopharyngeal nerves contain fibres which respond very selectively to mechanical stimulation, as stated be low The response to touch is, however much smaller in magnitude than that to chemical stimulation (Fig 1 P) The most sensitive area to touch seemed to be localized in the lateral sides of the palatal organ.

Facial nerve

The response to sapid solutions of the facial nerve in this fish was quite small, as in Fig. 2, I On the other hand, tactile stimulation caused relatively large response. There is no doubt, however that all taste substances which stimulated the palatal organ also were able to produce positive responses in the facial nerve, although to a minor degree. This smaller sensitivity might be attributed to the small amount of chemoreceptors located around the mouth. A smaller sensitivity of this nerve in response to sapid substances was also reported by HOAGLAND (1933) in catfish.

Branchial nerve

Typical records of the response from the branchial nerve innervating the chemoreceptors on gill rakers are reproduced in Fig. 2, II. All sapid substances which stimulated the palatal organ produced positive responses also in this nerve. Similarly to the glossopharyngeal nerve, the most effective stimulant was sugar Of the two peaks in the record A in Fig 2, II the later one was caused by the sugar solution flowing over those parts of the gill raker which were not stimulated when the solution was first poured out from pipette. Touch stimulation applied to the gill rakers produced a response with similar height as did chemical stimulation.

Nerve innervating the ventral fin of the bullhead (*Cottus quadricornis L.*)

Before testing, we have thought that the narrow ventral fins of this species may be capable of mediating chemical perception. It was found, l that the fins did not respond to any sapid substances at all, not even but served as very sensitive tactile organs (Fig 2, III)

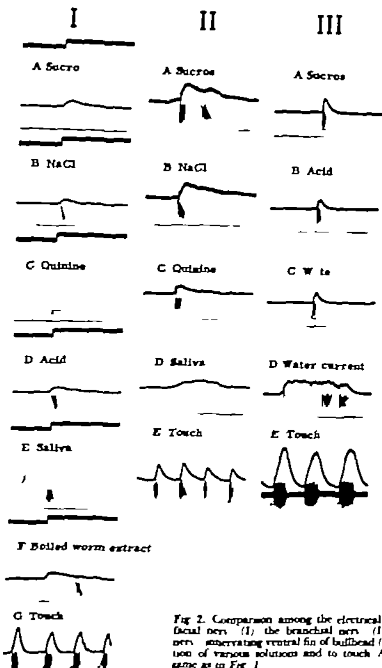


Fig 2. Comparison among the electrical responses from the facial nerv (I) the branchial nerv (II of carp, and the nerv innervating ventral fin of bullhead (III) to the application of various solutions and to touch Amplification is the same as in *Fig 1*

Single fibre analysis

General

From the experiments on fine strands of the glossopharyngeal nerve containing several active fibres, two types of fibre were distinguished One fibre type showed

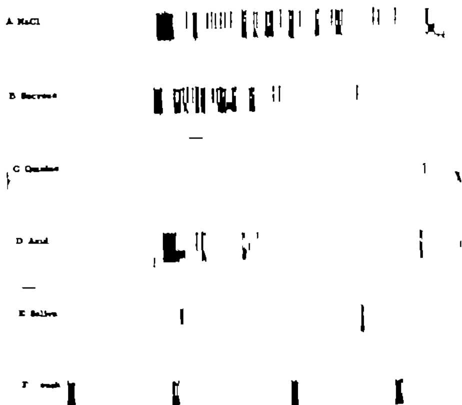


Fig 3. Response of single glossopharyngeal nerve fibre classified under the Type I (b) to the application of various solutions to the palatal organ. Record F responses of single touch fibre in the glossopharyngeal nerve to the tactile stimulations. A signal shows the moment of application of the test solution. Time in sec.

phasic activity and the other gave persistent activity In addition, it was often observed that some fibres did not display a continuous discharge, but showed grouped discharges as in Fig 4

None of the taste fibres tested responded to tactile stimulation to the palatal organ. The number of touch fibres seems to be relatively small compared with the chemoceptive fibres. HOAGLAND (1933) demonstrated in the catfish that the amplitude of the spikes from the facial nerve caused by touch is much larger than that of taste fibres, and this finding was confirmed by the present experiments on the facial nerve in the carp. Such a difference in spike height between touch and tast fibres could, however not be observed in the glossopharyngeal nerve of the carp (Fig 3 F) To discharge the touch fibres of the glossopha

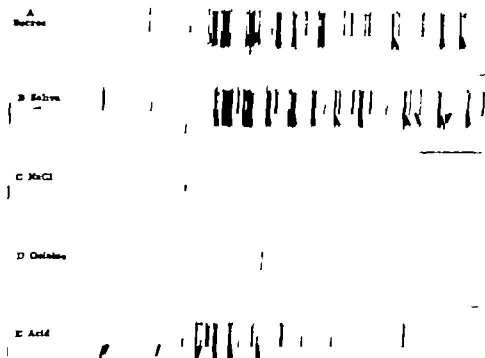


Fig. 4 Response of single glossopharyngeal nerve fibre classified under the Type III to the application of various solutions to the palatal organ. This is an example of grouped discharge. Time in sec.

ryngeal nerve, a stronger mechanical stimulation was needed than the flow of rapid solution over the palatal organ.

According to their response pattern, individual palatal chemoceptive fibres seem to be divided into seven groups as will be seen from Table I Among these different fibres the Types I II and III are the most common. Weak acid stimulated all types of taste fibres, except the majority of the salt fibres. Acetic acid in concentrations above 0 01 M (pH 3.2) was found to impair the sensitivity of chemoceptors to all kind of taste substances, or to cause a temporary lowering of the sensitivity

Type I

This is the fibre type which responds to many taste substances (NaCl sugar acid, glycerol and glycol) and even to human saliva, but not to quinine (Fig 5) Out of 37 such preparations, only 6 did not respond to saliva.

Type II

All kind of taste substances used, stimulated the fibres of this type but to

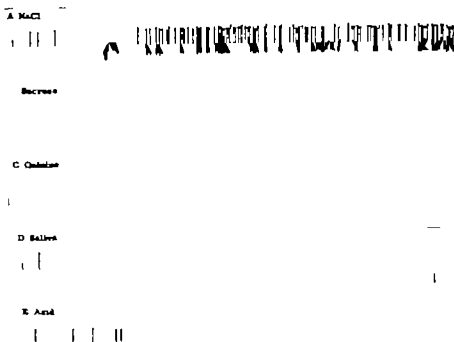


Fig. 5. Response of single glossopharyngeal nerve fibre classified under the Type IV to the application of various solutions to the palatal organ. Note the specific response to NaCl, and no response even to acid. Time in sec.

different degrees showing a great variety of individual response. In contrast to Type I the Type II fibres responded positively to quinine. Type I and II are the most unspecific taste fibres in the glossopharyngeal nerve of the carp.

Type III

This fibre type is characterized by its high sensitivity to sweet tasting substances, and was very frequently found in the palatal nerve (Fig. 4). It also responded to acid but did not respond to salt and quinine. Glycerol and glycol, which taste sweet to humans, produced positive responses in this fibre. Glycol was always more effective than glycerol, contrary to the results of behavioural studies reported by TAUBER (1929) showing no response to glycol in his fishes, though this might be due to the difference between species. Saccharine seems to have very little effect. It is particularly remarkable that the human saliva selectively stimulated these sweet fibres quite as strongly as sucrose, and in some cases even more so. Out of the 21 preparations, saliva failed to stimulate this fibre type in only two exceptional cases. As will be stated below the fibres of

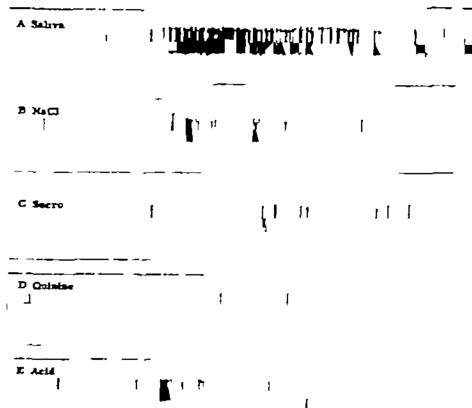


Fig. 6. Response of single glossopharyngeal nerve fibre classified under the Type VI the application of various solutions to the palatal organ Time in sec

the Type IV V and VII were never stimulated by saliva. On one occasion, the fibre responded slightly to salt in addition to sucrose, saliva and acid. The stimulating effects of the latter were, however by far stronger than that of the former On only one occasion, a functional single fibre was found which responded to sugar as well as to quinine.

Type IV

The fibres classified under this type responded primarily to salt solutions (Fig 5 It is interesting to note that among the 16 fibres of this type so far studied, all except two were not stimulated by acid at the concentration used. This is the only taste fibre type which was insensitive to weak acid.

Type V

These fibres responded both to quinine and acid and in this respect resembl some of the monkeys fibres (GORDON *et al* 1959 The number of such fibres seems to be relatively low

Table I

Stimulus / Fibre Type	NaCl (0.5 M)	Acetic acid (0.005 M)	Quinine (0.01 M)	Sucrose (0.5 M)	Saliva	Number of preparations
I	+	+	−	+	+	31
b	+	+	−	+		6
II	+	+	+	+	+	21
III	−	+	−	+	+	19
b.	−	+	−	+	−	2
IV a.	+	−	−	−	−	14
b	+	+	−	−	−	2
V	−	+	+	−	−	13
VI	−	(+)	−	−	+	3
VII	−	+	−	−	−	3

Type VI

Fibres which, surprisingly enough, showed a vigorous response only to saliva were but rarely found (Fig 6) They showed a very slight response to sucrose, salt and acid, and no response to quinine.

Type VII

Only three single functional fibres, which responded to no other test solutions than to weak acetic acid were found.

Tap water sometimes produced a response of fibres which were spontaneously active and which were silenced by the application of saliva, and such a spontaneously active fibre was silenced by the application of 0.5 M NaCl, while its activity was enhanced by the application of 0.5 M sucrose. In addition water sometimes also produced a moderately strong response when applied shortly after a previous application of acid as well as saliva. Repeated application of water however had no effect on these fibres, in contrast to the effect of repeated applications of water on the tongue of the frog or the cat.

Thresholds determined on the entire glossopharyngeal nerve for the different taste substances were low The salt threshold was less than 0.0005 M, that of sugar ranged from 0.01 M to 0 005 M (rarely 0.001 M) and that of quinine ranged from 0 0005 M to 0.0002 M.

Discussion

The palatal organ as well as the gill rakers display a differentiated chemical sensitivity as compared to that of the skin in general. This is in good accordance

with the dense distribution of taste buds in this organ and our finding of fibres responding to the application of sucrose and quinine solutions, which do not stimulate the endings of the common cutaneous nerve fibres.

A comparison of the chemoceptive response of the palatal nerves with that from the nerves innervating the lips, the barbels and the gill rakers gives us the impression that the palatal organ is the most important gustatory organ for feeding in this fish. It is quite interesting that although amphibian frog has "water" fibres, aquatic fish apparently does not. The fact that water in which fishes had been kept stimulates the chemoceptors while pure water does not implies a highly developed gustatory sensitivity in fish. Although we could not as yet identify the stimulants dissolved in this water, mucin and skin fluids possibly may serve as the effective agents. In this connection it is interesting to note the behavioural observations demonstrating that fishes are able to detect the presence of another species or of wounded fish.

The integrated responses to salts were usually rather smaller in magnitude than those to sugar and even to saliva. We cannot, however, argue about the quantitative comparison of the integrated responses, because the magnitude of the response is influenced not only by the number of active fibres and their impulse frequencies but also by the size and configuration of the individual spikes.

The existence of many fibres displaying a broad gustatory spectrum, as was described above, directs the attention to the possibility of a simple osmotic effect as the primary mechanism in producing taste sensation in the fish. This possibility, however, is ruled out because sugars are more effective than many electrolytes.

Among the taste substances which stimulate the palatal organ, the most powerful is acid and next to that sugar. Specific "acid" fibres seemed to be few in total number, but acid stimulates the majority of the gustatory fibres.

The effect of human saliva is rather interesting. In spite of being tasteless to humans, saliva vigorously stimulated many kinds of fibres in the fish, including sweet fibres which were sometimes stimulated even stronger than by sugar. To our surprise, we even found a few fibres specifically responding to saliva. Saliva, of course, contains many substances, such as proteins (including an enzyme and mucin), salts etc. But saliva does not stimulate salt fibres. It is rather difficult to identify the effective substances because of the complicated nature of saliva, and this problem will be dealt with in a subsequent paper. The extract of mucous substances covering the body surface of the fish also stimulated the taste nerves, as already described. It is notable that the sweet fibres were found to be stimulated by the mucous substances as well as by saliva.

It is obvious that the great majority of taste fibres running to the palatal organ of the carp display a rather broad gustatory spectrum. This raises again the question previously brought up by PFAFFMANN (1941), as well as by one of us, whether a taste fibre might possess in its endings different receptors re-

sponding to different classes of gustatory stimuli. As it is difficult to find any common denominator for the excitatory effect of such stimuli as sugar, salt, quinine and acid, it seems more likely that the broad gustatory sensitivity of these fibres is due to the existence of different chemosensitive sites in the ending fibrilles of the individual gustatory nerve fibre. Such an arrangement would thus be similar to Granit's system of dominators in the retina. Strictly specific taste fibres, corresponding to Granit's modulators, we have found only to salt (Type IV a, 14 fibres) and to acid (Type VII, only 3 fibres) and to human saliva (Type VI, only 3 fibres). In addition to these we have the Type V fibres (13 fibres), which respond only to quinine and acid. From these findings it is possible to picture a mechanism enabling the fish to elaborate a fairly high level of gustatory discrimination. Behavioural studies on fresh water fishes have also revealed that these fishes discriminate between the four basic classes of tastes (UCHIDA 1933). Further, the high sensitivity of the palatal organ to human saliva, as well as to the mucous covering the scales of fish, would suggest that the taste of these fishes may serve its specific purposes, in addition to their olfactory mechanism, in the search for food and in detecting other individuals.

Our finding of a relatively great number of taste fibres responding to sweet tasting substances is in close agreement with behavioural experiment on fresh water fishes. TRUDEL (1929) made an extensive study of the taste reactions of minnows (*Phoxinus laevis*). He determined the threshold concentration for saccharose to 0.01—0.005 M, while UCHIDA (1933) gave a value as low as 0.0002 M. The threshold of 0.01—0.005 M which we found for the electrical response is thus in good agreement with Trudel's figures. The threshold for sucrose in man is generally given as 0.01 M which implies that the carp's sensitivity for "sweet" may be equally strong as that of humans. The lower threshold found by UCHIDA may depend on individual differences as well as on the age of the fish. We have every reason to suppose that there may exist as much of species and individual differences in fish as there are between mammals and it is well known that a pike does not snatch at a bait of sweet dough as does a minnow or a carp.

References

COHEN, M. J., S. HAGIWARA and Y. ZOTTERMAN, The response spectrum of taste fibres in the cat. A single fibre analysis. *Acta physiol. scand.* 1955. 33. 316—332.

GORDON, G., R. KITCHELL, L. STRÖM and Y. ZOTTERMAN, The response pattern of taste fibres in the chorda tympani of the monkey. *Acta physiol. scand.* 1959. 46. 119—132.

HOAGLAND, H., Specific nerve impulses from gustatory and tactile receptors in catfish. *J. gen. Physiol.* 1933. *16.* 685—693.

PFAFFMANN, C., Gustatory afferent impulses. *J. cell. comp. Physiol.* 1941. *17.* 243—258.

TRUDEL, P. J., Untersuchungen über Geschmacksreaktionen der Fische auf süsse Stoffe. *Z. vergl. Physiol.* 1929. *10.* 367—409.

UCHIDA, Y., Studies on the taste of fish (in Japanese). *Kagaku* 1933. *3.* 196—200.

Acta physiol. scand. 1961 52. 162—166

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The Role of the Adrenals on the Mucosal Mast Cells and Tissue Eosinophils in the Gastric Wall of Rat

By

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Abstract

RÄSÄNEN T *The role of the adrenals on the mucosal mast cells and tissue eosinophils in the gastric wall of rat.* Acta physiol. scand. 1961 52 162—166. — The effect of ACTH on the mucosal mast cells and tissue eosinophils in the gastric wall of adrenalectomized and intact rats was studied. In intact rats, ACTH caused almost complet degranulation of the mucosal mast cells and a highly significant fall in tissue eosinophilia. Its degranulating effect on the mucosal mast ells and its tissue eosinopenic effect were inhibited after adrenalectomy Adrenalectomy alone caused a slight increase in both these cell types in the gastric mucosa. The significance of mast cells as mucosal histamine *depot and its possible primary role as secretory stimulant of the* parenchyma and secondary rol in causing reduction of tissue eosinophilia is discussed.

The degranulation of the mucosal mast cells in the gastric mucosa of rat is a rapid and quantitative process under *the influence of glucocorticoids.* Mineralocorticoids do not seem to ha e a degranulating effect on these cells (RÄSÄNEN 1961) The location of mucosal mast cells in the superficial part of the gastric mucosa where the histamine content is greatest (FELDBERG and HARRIS 1953) suggests that histamine conjugated with the polysaccharides of the mast cell granules constitutes the histamine depot of the gastric mucosa.

The degree of tissue eosinophilia in the lamina propria of the stomach may increase (WESOLIUS and TERA 1958) or decrease (RÄSÄNEN 1960 a) after a glucocorticoid njection, depending presumably on the rate of eosinophil consumption in the reactions stimulated in the lamina propria and on the compensating capacity of myeloid and possible local eosinopoesis. Histamine released from the tissues under the influence of glucocorticoids (SCHAYER et al. 1954) causes a drop in blood eosinophiles immediately (DUNER and PER OW

Table I Number of mucosal mast cells and tissue eosinophils ± standard error per sq.mm. in the gastric wall of intact and adrenalectomized rats with and without ACTH-therapy

	Adrenalectomized		Intact	
	ACTH	Saline	ACTH	Saline
Mast cells	911 ± 101	1,345 ± 122	4.8 ± 1.9	945 ± 121
Eosinophils	2,824 ± 99	3,564 ± 301	247 ± 65	2,550 ± 158

1958 ROST et al. 1958) or by occasioning the elease of endogenous histamine through stimulation of the pituitary-adrenal system (HALPERN and BÉKOS 1951 LECOMTE et al. 1959 ZARATZIAN 1959)

The conjugation of glucocorticoids takes place to a great extent in the gastrointestinal canal — the reaction is inhibited in deintestinalized rats and is retarded when the portal circulation slows down in hepatic cirrhosis. It has been established that n the latter case the eosinopenic response to Thorn s test is also reduced (WAHI and RAMACHANDRAN 1958)

ACTH has been established earlier to have a degranulating effect on mucosal mast cells and a reducing influence on tissue eosinophilia (RÄSÄNEN 1960 a b) This effect probably occurs through stimulation of the adrenal cortex. The mucosal mast cell count must perhaps be expected to rise after adrenalectomy when corticoid secretion is absent and the tissue eosinopenic effect is inhibited

Method

Mal rats, age 4 months and weight 146—220 g, of Dawley-Sprague strain were used in the investigation. Adrenalectomy was performed on 20 rats after the animals were adapted to the conditions in the laboratory Ten adrenalectomized and 10 intact rats were given 4 IU of ACTH (Cortrophine Z, Organon) intramuscularly once day for 10 days. Ten adrenalectomized and 11 intact rats were given 0.5 ml saline injections daily for the same period. Food and water were given ad libitum the adrenalectomized animals were given saline to drink. Decapitation was performed 24 hours after the last injection during which time the rats fasted and were kept in cages with net floor

The rat stomachs were removed immediately after decapitation and split longitudinally in two. The halves were fixed in formalin and in basic lead acetate. The sections, staining and cell count were performed as reported earlier in detail (RÄSÄNEN 1960 a, b)

Results

The cell count is given in cells per square millimetre of tissue. The values are shown in T ble I and Fig 1

Metachromatically granulated mucosal mast cells disappeared almost completely in intact rats given ACTH ($P < 0.001$) probably owing to their degranulation. No degenerative changes were demonstrable in the nuclei of even extensively degranulated mucosal mast cells. A corresponding degranulation under the influence of ACTH was not established in the submucosal mast cells.

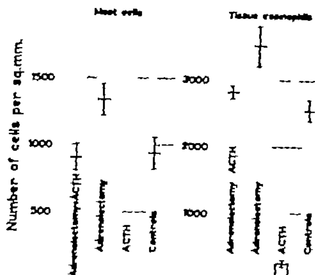


Fig. 1. Diagram of the amount of mucosal mast cells and tissue eosinophils in rat stomach wall after adrenalectomy and ACTH application.

The degranulating effect of ACTH on mucosal mast cells seemed to be inhibited after adrenalectomy ($P < 0.02$). Although it was possible in this group to count mast cells in the stomach of two rats only in which no superficial mucosal lesions occurred, their mucosal mast cells did not reveal even partial degranulation or vacuolisation, both of which were so profuse in the mucosal mast cells of the intact rats treated with ACTH. The amount of mucosal mast cells increased ($P < 0.05$) after adrenalectomy and their granulation was intensive.

Tissue eosinophilia diminished very distinctly ($P < 0.001$) during ACTH therapy in the basal part of the lamina propria. This phenomenon disappeared after adrenalectomy and even changed to a slight increase ($P < 0.05$) in adrenalectomized rats.

Discussion

The increase in the amount of mucosal mast cells and the intensification of their granulation probably indicate that the consumption of polysaccharides, containing glucuronic acid, under the influence of endogenous glucocorticoids in the intact animals is inhibited after adrenalectomy. The mucosal stores of mast granules which are augmented in this connection are perhaps able to bind greater quantities of histamine. The amount of histamine increases after adrenalectomy especially in the gastrointestinal canal (BARTLET and LOCKETT 1959).

ACTH degranulated the mucosal mast cells fairly thoroughly during the experimental period of 10 days. This process obviously took place through the mediation of adrenocortical stimulation. It was attributed to the mobilisation of glucocorticoids since no comparable phenomenon was produced by mineralocorticoids (RÄSÄNEN 1961).

Glucocorticoids stimulate gastric secretion (GRAY et al. 1951 PLADOS & PHILIPPU 1958, PEREMANS 1958) and also histamine liberators (PATON and SCHACHTER 1951 PICCALUGA et al. 1958) The stimulating effect of histamine on gastric secretion is inhibited in Addison's disease (THORN et al. 1951) It is possible that the stimulation of the mucosal parenchyma takes place through the elease of the histamine of the mucosal mast cells. This stimulation is probably so immediate in the mucosa that antihistamines are incapable of inhibiting it (HALPERN and MARTIN 1946) for instance, the stimulation occurring by this route is increased by aminoguanidine (IVY and LIEPINS 1960) a histaminase inhibitor Gastric secretion is inhibited by hibernation (GILLESPIE 1956) The degranulation of the mast cells (HÖGBERG and UVNÄS 1957 UVNÄS et al. 1960) and the histamine release (SZILÁGYI et al. 1960) are also inhibited in the same connection.

ACTH caused a fairly complete loss of tissue eosinophilia. This loss obviously occurs also through the stimulation of the adrenal cortex. The drop in blood eosinophilia is dependent on the amount of ACTH (SPEIRS et al. 1959) and tissue eosinophilia of rat stomach decreases in correlation with the amount of glucocorticoids (RÄSÄNEN 1961) Tissue eosinopenia emerges, however more slowly than the corresponding degranulation of the mucosal mast cells. Prolonged ACTH therapy causes in man retention of eosinophils in the blood and inhibition of response to Thorn s test (MIROZZI 1959) probably because of the diminution of the polysaccharide-histamine depot of the tissues.

Blood eosinophilia is lowered in intact and adrenalectomized rats by injecting a foreign serum, gammaglobulins and many aminoacids (ASCHKENASY 1959) which are histamine liberators (PATON 1957) into the great mast cell depot of the abdominal cavity The same chain of events probably occurs in the gastric mucosa under the influence of ACTH-stimulated glucocorticoids conjugation of glucocorticoids — release of histamine — eosinopenia. Stimulation of the parenchyma is associated with this chain of events.

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References

ASCHKENASY A., Some new experimental data on the role of the adrenals in the regulation of blood eosinophilia. Ann. N Y Acad. Sci. 1959. 77 574—588.

BARTLETT A. L. and M. F LOCKETT Effect of adrenalectomy on tissue histamine in rats. J Physiol. (Lond.) 1959 147 51—57

DUNER, H. and B. PERNOW Histamine and leucocytes in blood during muscular work in man. Scand. J clin. Lab Invest. 1958. 10 394—399

FELDBERG, W and G. W HARRIS, Distribution of histamine in the mucosa of the gastric intestinal tract of the dog. J Physiol. (Lond.) 1953. 120 352—364.

GILLESPIE, J A., The effect of lowered body temperature on histamine-induced gastric secretion. Quart. J exp. Physiol. 1956. 41 290—294

GRAY S. J J A. BENSON, R. W REIFENSTEIN and H. M. SPIRO, Chronic stress and peptic ulcer

I Effect of corticotropin (ACTH) and cortisone on gastric secretion. *J Amer med. Ass.* 1951 *147* 1529—1537

Halpern, B. N. et S. A. Binon, Suppression de l'effect éosinopénique de l'histamine par l'hypophysectomie chez le rat. *C. R. Soc. Biol.* (Paris) 1951 *145*. 531—533.

Halpern, B. N. et J Martin, Production expérimentale d'ulcus et de perforation gastriques par des doses massives d'histamine chez le cobaye protégé par des antihistaminiques. *C. R. Soc. Biol.* (Paris) 1946. *140*. 830—832.

Högberg, B. and B. Uvnäs, The mechanism of the disruption of mast cells produced by compound 48/80. *Acta physiol. scand.* 1957 *41* 345—369.

Ivy A. C. and K. W Lienon, Effect of 1,2,4-triazole ethylamine on gastric secretion. *Amer J Physiol.* 1960. *198*. 614—618.

Lecomte, J., H. van Cauwenberge et M. Palem-Vliers, Stimulation corticosurrénalienne par libération de l'histamine endogène chez l'homme. *Acta allerg* (Kbh.) 1959. *14* 291—293.

Minozzi, A., Trattamento protratto con ormone adrenocorticotropo ad attivia ritardata comportamento dell'eosinofilia emetica. *Gazz. med. ital.* 1959. *118*. 331—336.

Paton, W A. D., *Histamine release by compounds of simple chemical structure. (A review.) Pharmacol. Rev* 1957 *9*. 269—328.

Paton, W A. D and M. Schachter, The influence of an antihistamine drug on the release of histamine in the unanaesthetized dog *Brit. J Pharmacol.* 1951 *6*. 509—513.

Perlmann, J., L'influence de la prednisolone sur la muqueuse gastrique du rat blanc surrénalectomisé ou non. *Acta gastro-ent. belg* 1958. *21* 665—673.

Piccaluga, A. J., E. Machado, A. Paula, H. Orforo y A. Kaldrov Estudio comparativo entre la accion de la histamina y la reserpina sobre la acidimetria y la mucosa gastrica. *Pren. méd argent.* 1958. *45*. 2143—2148.

Plaidon, T C. and A. J Phillippo, The effect of prednisone on gastric secretion and urine and uropepsin excretion in the dog *Gastroenterology* 1958. *35*. 183—189

Räsänen, T., Long term effect corticotropin, cortisone, and growth hormone on the tissue eosinophils in rats stomach. *Acta endocr* (Kbh.) 1960. *33*. 370—374

Räsänen, T., Mucosal mast cells of rat stomach Influence of ACTH, cortisone and growth hormone. *Gastroenterology* 1960. *38*. 70—75.

Räsänen, T Quantitative effect of some steroid hormones on the mucosal mast cells and tissue eosinophils in the rat stomach wall. *Acta endocr* (Kbh.) 1961 *37* 153—158

Rosa, L., G. C. Ceracchi et G. Bertoani, Les variations numériques des éosinophiles circulants après injection d'antigène, d'histamine ou de 5-hydroxytryptamine *Acta allerg* (Kbh) 1958. *12*. 427—431

Schayer, R. W R. L. Smiley and K. J Davis, Inhibition by cortisone of binding of new histamine in rat tissues. *Proc. Soc exp Biol.* (N. Y) 1954 *87* 590—592.

Speirs, R. S., H. Panzenhagen and J. Sullivan, Assay of ACTH activity based on the eosinopenic response in normal and hypophysectomized mice *Endocrinology* 1955. *57* 300—310.

Szilágyi, T., A. Kovács and B. Csaba, Effect of hypothermia on histamine release induced by epinephrine infusion. *Amer J Physiol.* 1960. *199*. 272—274

Thorn, G W., P H. Forsham and K. Emerson, *The diagnosis and treatment of adrenal insufficiency* 2. ed. Charles C. Thomas, Springfield, III 1951

Uvnäs, B., B. Diamant B. Högberg and I. L. Thon Mechanism of mast-cell disruption induced by principle extracted from ascaris suis. *Amer J Physiol* 1960. *199* 575—578.

Wahi, P N and S. Ramachandran, *Adrenal cortical function in portal cirrhosis. A M A. Arch. Path.* 1958. *65* 482—486

Westphal, O. and H. Tesh, The effect of cortisone adrenalectomy and hypophysectomy on the tissue eosinophil count in the glandular stomach of the rat. *Acta endocr* (Kbh.) 1958. *28* 197—204.

Zarafonetis, C. L., The role of histamine in drug-induced eosinopenia and the blocking effects of tripelennamine hydrochloride *J exp. Pharmacol.* 1959 *1* 628—637

Acta physiol. scand. 1961. 52. 167—170

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Uptake of Dopamine by the Storage Granules of the Adrenal Medulla in Vitro

By

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Abstract

BERTLER, Å., G. HALL, N.-Å. HILLARP and E. ROSENGREN. *Uptake of dopamine by the storage granules of the adrenal medulla in vitro.* Acta physiol. scand. 1961. 52. 167—170. — Large amounts of dopamine are taken up by the amine storage granules of the cow adrenal medulla when the granules are incubated at + 31° in a solution containing dopamine. At this temperature the amine accumulates even against an apparent concentration gradient but at 0° only small amounts — if any at all — are incorporated. The amine seems to be bound in the granules but is released on osmotic lysis of the granules.

Dopamine (DA) is rapidly taken up the amine storage granules of the adrenal medulla when formed *in vivo* in the cells (BERTLER, HILLARP and ROSENGREN 1960). It has now been found that these granules are capable of incorporating DA also *in vitro*.

Material and Methods

Amine granules from cow adrenal medulla were isolated in 0.3 M sucrose as described previously (HILLARP 1958). They were not washed and were thus contaminated with other cell particles (i.e. mitochondria) and, to some extent, also with cytoplasmic sap. Immediately after their isolation the granules (100—150 mg wet weight/ml) were suspended in a solution of DA — HCl (10—20 μmoles/ml) containing (in μmoles/ml) ascorbic acid 20, potassium chloride 40, magnesium chloride 5, sodium phosphate (pH 7.5) 15 and sucrose 180. The pH was adjusted to 7—7.5 with potassium carbonate immediately before the use of the solution. Incubation was performed without shaking at + 31° for one hour, after which the suspension was chilled to 0°, diluted 40 times with

Table I. Total amounts of catecholamines in the supernatant (Sup.) and in the granule sediment (Gran.) after incubation of amine granules in a medium containing DA.
After the incubation the granule suspensions were either directly centrifuged or first diluted 40 times with 0.5 M sucrose (or 0.1 M sucrose for osmotic lysis) and then centrifuged.

Incubation temperature	Treatment after the incubation	Cell fraction	A µg	NA µg	Changes in A + NA µg	DA µg	A + NA + DA µg
0	No dilution	Sup.	175	100		1,570	
		Gran.	2,030	1,300		165	3 700
0°	Diluted 40 ×	Gran.	2,050	1 400		70	3,500
+ 31	No dilution	Sup.	605	280	+ 610	830	
		Gran.	1 750	1,220	− 580	595	3,550
+ 31	Diluted 40	Gran.	1,650	1,150	− 650	460	3,250
+ 31	Osmotic lysis	Gran.	13	70		20	

cold 0.5 M sucrose and centrifuged at 38,000 × g for 30 min. The catecholamine content of the granule sediment and of the supernatant was determined spectrophotofluorimetrically (BERTLER, CARLSSON and ROSENGREN 1958, CARLSSON and WALDECK 1958). To obtain pure amine granules, density gradient centrifugation of granules incubated as described above was made by a method previously described (HILLARP 1958 a).

Results and Discussion

Large amounts of DA — up to about 20 per cent of the amounts of adrenaline (A) + noradrenaline (NA) in the granuels — were regularly recovered in the granule sediment after an incubation at + 31° for one hour. The data from a typical experiment are found in Table I. That this "bound" DA had in fact been taken up by the amine storage granules was shown by means of density gradient centrifugation. The DA showed the same distribution as that of the storage granules. About 50 per cent of the DA as well as of the A + NA was recovered in the sediment containing pure amine granules with the highest density, in which the amines are normally stored together with an equivalent amount of adenosinephosphates (cf. HILLARP 1960 a).

The experiments indicate (see Table I) that DA — just as A and NA — can penetrate the granule membrane from without at 0° and reach about the same concentration in the intragranular water as in the medium. At this temperature, however, only small amounts — if any at all — of DA were incorporated in agreement with previous findings (CARLSSON and HILLARP 1958, HILLARP 1959). The fact that the storage process is inhibited at lower temperatures does not necessarily mean that it is an active, energy-utilizing process. It is in

fact equally probable that this phenomenon is caused by a change in the state of the storage complex (cf. HILLARP 1960 b). At 0° the complex may well be more "condensed" and thus prevent amines from penetrating it.

The experiments clearly show that at + 31° the granules are capable of accumulating DA even against an apparent concentration gradient. The incorporated DA is "bound" in such a way that it is not released but held for at least 24 hours if the granules are kept suspended in 0.3 M sucrose at 0° in spite of a very high difference of DA concentration (more than a hundredfold) between the granules and the medium. Since it does not seem possible that an active transport mechanism is responsible for the maintenance of the steep concentration gradient, it seems reasonable to assume that the amine is kept in a bound state, perhaps in a way similar to that of the stored A and NA. There is another striking similarity between the ways in which the three amines are stored, since the DA — just as the A and NA — is immediately released on osmotic lysis of the granules (Table I).

Experiments with incubation media of different compositions have not supported the view that ascorbic acid, magnesium chloride etc., which usually were included, are essential for the amine incorporation. Adenosinediphosphate (15 μ moles/ml) had no obvious effect. In fact, DA was readily taken up from a solution containing only DA—HCl in 0.3 M sucrose (neutralized with potassium carbonate).

Even when large amounts of DA were incorporated in the granules, no increase in the total amount of stored amines was observed (Table I). On the contrary, a loss usually occurred. This is easily understood on the basis of the fact that the stored A and NA are released when the granules are kept at elevated temperatures (HILLARP 1958 b). In the present work it was found that this "spontaneous" release is not materially influenced by the presence of DA in the suspension medium. Thus it is not possible at present to decide whether the storage of DA is a result of an exchange between this amine and the stored A and NA, and not even whether the two processes, storage of DA and release of A and NA, are coupled together.

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References

BERTLER, Å., A. CARLSSON and E. ROSENGREN, A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* 1958. *44.* 273—292.

BERTLER, Å., N.-Å. HILLARP and E. ROSENGREN, Storage of new-formed catecholamines in the adrenal medulla. *Experientia* (Basel) 1960. *16.* 419.

CARLSSON, A. and N.-Å. HILLARP, On the state of the catecholamines of the adrenal medullary granules. *Acta physiol. scand.* 1958. *44.* 163—169.

CARLSSON A. and B. WALDECK, A fluorimetric method for the determination of dopamine (3-hydroxytyramine) *Acta physiol. scand.* 1958, *44* 293—298.

HILLARP N. Å., Isolation and some biochemical properties of the catecholamine granules in the cow adrenal medulla. *Acta physiol. scand.* 1958 a. *43* 82—96.

HILLARP N.-Å., The release of catecholamines from the amine containing granules of the adrenal medulla. *Acta physiol. scand.* 1958 b. *43* 292—302.

HILLARP N.-Å., Further observations of the stat of the catecholamines stored in the adrenal medullary granules. *Acta physiol. scand.* 1959. *47* 270—279

HILLARP N.-Å., Different pools of catecholamines stored in the adrenal medulla. *Acta physiol. scand.* 1960 a. *50.* 8—22.

HILLARP N. Å., Catecholamines: mechanisms of storage and release. Adv Abstr *Symp.* Lect. First Int. Congr Endocrinol. Copenhagen 1960. *Acta endocr.* Sept. 1960 b. *181*—185.

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The Fate of Uniformly Labelled C Fructose in Different Parts of the Brain and the Pituitary in Goats

By

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Abstract

LARSSON, S. *The fate of uniformly labelled ¹⁴C fructose in different parts of the brain and the pituitary in goats.* Acta physiol. scand. 1961 52 171—177 — The fate of uniformly labelled ¹⁴C fructose has been studied in different parts of the brain and in the anterior and posterior pituitary Generally the metabolism of fructose was less than that of glucose. Particularly in the anterior part of the pituitary the fructose utilisation was small. As the oxygen consumption was little affected by the fructose the utilisation of endogenous substances must be increased compared to experiments where glucose was used as substrate. The rate of conversion of fructose into various amino acids was considerably lower in the periventricular parts of the hypothalamus and in the anterior part of the pituitary as compared to the glucose experiments. The observed differences are discussed in relation to the importance of the pentose shunt.

BELOFF-CHAIN *et al.* (1955) investigated the fate of ¹⁴C-labelled fructose in slices of rat brain by means of quantitative radio-chromatography No differences were found except for a generally lower metabolism as compared to glucose. Further previous studies have shown that marked differences exist in the fate of ¹⁴C-labelled glucose in various parts of the brain and in the anterior and posterior parts of the pituitary (CHAIN, LARSSON and POCCHIARI 1960 ANDERSSON, LARSSON and POCCHIARI 1961)

The present study was undertaken to see if the various parts of the brain and the pituitary also showed the above mentioned differences when fructose was used instead of glucose.

Table I. Oxygen uptake, CO_2 and lactic acid formation in different parts of the brain and the pituitary after incubation with ¹⁴C fructose or ¹⁴C glucose

Results expressed as μl of oxygen uptake per 25 mg of tissue (wet wt.) after 1 h incubation in O_2 at 37° C. CO_2 and lactic acid expressed as μg fructose or glucose converted, based on the fraction of total radioactivity of fructose or glucose incorporated under the conditions mentioned above. Fructose or glucose concentration 0.1 %; total radioactivity 10 μC per vessel. Mean values ± s.e.m. Values within brackets derived from Andersson et al. (1961).

Differences statistically significant $p < 0.05$

Tissue		O	CO_2	Lactic acid	No. of exper
Periventr. area	Fr	35.3 ± 2.6	4.5 ± 0.5	31.1 ± 2.0	5
	GL	(38.7 ± 3.1)	(11.9 ± 1.0)	(102.3 ± 7.2)	14
Ventromed. area	F	32.2 ± 4.0	4.9 ± 1.4	38.6 ± 2.9	5
	GL	(34.0 ± 3.3)	(10.8 ± 1.9)	(83.8 ± 4.3)	10
Cerebral cortex	Fr	33.0 ± 4.1	9.2 ± 1.6	23.4 ± 2.2	5
	GL	36.2 ± 3.7	10.5 ± 1.4	103.3 ± 9.7	6
Posterior pituitary	F	26.2 ± 3.3	5.2 ± 1.3	33.3 ± 2.4	5
	GL	30.3 ± 3.2	(8.7 ± 1.6)	(72.5 ± 5.1)	10
Anterior pituitary	F	20.1 ± 1.7	0.4 ± 0.1	5.3 ± 1.8	6
	GL	(26.1 ± 2.6)	(2.3 ± 0.3)	(30.3 ± 1.3)	8 & 9

– = differences statistically significant
GL = glucose, F = fructose

Methods

Generally labelled ¹⁴C fructose and ¹⁴C glucose were obtained from the Radiochemical Centre, Amersham, England. The radioactive material was diluted to give a specific activity of 20 μC per mg.

The incubation medium was the same as that used by Andersson *et al.* (1961): phosphate buffer with the radioactive material 0.0056 M, containing 3.6 mC mmole at pH 7.3. Adult female goats fed *ad libitum* were used in the experiments. The animals were killed by decapitation and the samples dissected out as quickly as possible.

The following samples from the brain and the pituitary were taken:

1. Periventricular tissue
2. Ventromedial tissue
3. Parts of the posterior pituitary
4. Parts of the anterior pituitary
5. Parts of the cerebral cortex, taken medially just caudal to the frontal lobes.

Parts 1—4 included the same areas as described earlier (Andersson *et al.* (1961)).

After incubation of the tissue for one hour at 37° C — the gas phase being pure oxygen — the samples were treated in exactly the same way as previously described (Crane *et al.* 1960, Andersson *et al.* 1961). As in the previous experiments the oxygen consumption was measured every 15 min. After incubation the former Na_2CO_3 was collected and precipitated with $BaCl_2$ as described by Villee and Hastings (1949).

Table II. Amino acid formation from metabolized ^{14}C fructose or ^{14}C glucose in different parts of the brain and the pituitary

Results expressed as μg fructose or glucose converted per 25 mg of tissue (wet wt.) after 1 h incubation in O_2 at 37° C. Fructose or glucose concentration 0.1 % total radioactivity 10 μc per vessel. Mean values ± s.e.m. Values within brackets derived from Andersson et al. (1961).
Differences statistically significant $p < 0.05$

Tissue		Alanine	Aspartic acid	Glutamic acid	GABA	Glutamine	Arginine
Periventr. area	F	0.9±0.1	0.5±0.1	2.9±0.4	2.2±0.3	0.6	0.3±0.1
	Gl.	(0.6±0.1)	(0.9±0.1)	(6.0±0.7)	(3.5±0.3)	(1.2±0.3)	(0.9±0.2)
Ventromed. area	F	1.0±0.02	0.7±0.2	3.2±0.4	2.3±0.3	0.6	trace
	Gl.	(1.3±0.3)	(0.5±0.1)	(4.4±0.6)	(2.6±0.5)	(1.1±0.2)	(0.4±0.1)
Cerebral Cortex	F	0.6±0.1	1.1±0.4	4.0±0.8	1.0±0.3	0.4	trace
	Gl.	1.4±0.5	0.8±0.2	6.4±0.8	1.3±0.3	1.5±0.4	trace
Posterior pituitary	F	0.6±0.1	0.5±0.1	4.4±0.6	0.8±0.2	0.3	0.3±0.1
	Gl.	(0.7±0.2)	(0.8±0.1)	(4.1±0.5)	(1.0±0.03)	(0.7±0.1)	(0.7±0.2)
Anterior pituitary	F	0.1	trace	0.1	—	0.2	0.2
	Gl.	(0.9±0.1)	(0.4±0.03)	(0.7±0.1)	—	(0.9±0.2)	(0.4±0.05)

= differences statistically significant
Gl. = glucose Fr. = fructose GABA = γ-aminobutyric acid
Number of experiments the same as in Table I.

The radioactive metabolites formed from the glucose and fructose were separated by paper-chromatography as described earlier (Craig *et al.* 1960). All chromatograms were scanned quantitatively by a modification of the automatic scanning device according to Frank *et al.* (1959).

Results

Table I shows the oxygen consumption and the formation of CO_2 and lactic acid in different parts of the brain and the pituitary when the samples were incubated with ^{14}C fructose or ^{14}C glucose. The "fructose"-values were generally lower than the "glucose" ones. Except for the values on the oxygen consumption the differences were statistically significant. When the tissue was incubated with fructose the oxygen consumption was lower than when incubated with glucose. In the hypothalamic samples and the tissue from the posterior pituitary the decrease in CO_2 and lactic acid production was essentially the same when fructose was present in the substrate. Thus, the CO_2 formation for these three parts was only half, and the lactic acid formation about one third of the values found for glucose. In the experiments on the anterior pituitary the corresponding differences were considerably larger. In the cerebral cortex the decrease when using fructose was most pronounced in the lactic acid production.

Table III The occurrence of free unchanged ^{14}C fructose or ^{14}C glucose in the cells of different parts of the brain and the pituitary

Results expressed as μg fructose or glucose per 25 mg of tissue (wet wt.) after 1 h incubation in O_2 at 37° C. Fructose or glucose concentration 0.1 %, total radioactivity 10 μC per vessel. Mean values ± s. e. m.

Tissue	Incubation medium		
	^{14}C fructose		^{14}C glucose
	Free fructose	Glucose formed from fructose	Free glucose
Periventricular area	8.3 ± 1.2	0.2	8.3 ± 1.3
Ventromedial area	7.3 ± 0.8	0.3	3.3 ± 0.4
Cerebral cortex	6.7 ± 0.8	0.4	5.8 ± 0.7
Posterior pituitary	15.3 ± 2.5	0.9	8.3 ± 1.4
Anterior pituitary	11.5 ± 1.5	0.3	12.0 ± 2.3

Table II shows the amounts of ^{14}C fructose and ^{14}C glucose converted into the various amino acids, found after extraction of the samples from the different parts of the brain and the pituitary. Generally there was a decreased total amino acid formation from the fructose in the medium as compared to glucose. This was particularly evident in the samples from the anterior pituitary and from the periventricular tissue. In these parts the amino acid formation from the fructose in the medium was so small that in the anterior pituitary not more than traces could be found. There was a tendency for lowered conversion of fructose into glutamic acid and γ-aminobutyric acid in all samples studied (except for glutamic acid in the posterior pituitary). The formation of alanine and aspartic acid did not seem to be changed when fructose was present in the medium compared to glucose. Due to analytical difficulties in separating ^{14}C *fructose and* ^{14}C *glutamine chromatographically the* values of the amount of fructose converted into glutamine were not as reliable as desired. It seemed, however, that the amount of glutamine found in this case was less than the amount formed from glucose. Table III shows the tendency for a greater accumulation of the fructose in the cells as compared to the glucose experiments. The same table also shows that ^{14}C glucose can be formed and found in the cells after incubation of the samples with ^{14}C fructose. In addition some glucose was also found in the incubation medium in these experiments. Generally the amount of unchanged fructose found in the cells after incubation was about 20 *of the total amount of fructose disappearing from the medium.*

The radioactivity found in the insoluble residues after incubation with fructose were not significantly different from those formed with glucose.

Discussion

McIlwain (1953 a, b) studying the possible role of fructose as a substrate for th brain, found that the respiratory rate of cerebral cortex with equimolar concentrations of glucose or fructose was higher with glucose than with fructose, provided the concentrations of the two substrates were equivalent to 5 mM or less. In the present experiments where parts other than the cerebral cortex were also studied similar observations were made. While the respiratory exchange of the cerebral cortex was only little affected by fructose in the present experiments, the differences between fructose and glucose were more pronounced in the other parts studied with the exception of the posterior pituitary This might imply that the hypothalamus and the anterior pituitary in particular are more dependent on the endogenous supply of oxidizable material when fructose is the substrate than when glucose is present in the incubation medium. In this connection, however it is of interest to note that the $^{14}CO_2$ formation of the anterior pituitary in relation to oxygen consumption is also very low when glucose instead of fructose is present in the substrate (Table I and Andersson et al. 1961) From the values obtained from the glucose experiments it is further evident that *in vitro* more than 50 % of the CO_2 formed is derived from endogenous oxidation. However fructose will generally increase the endogenous oxidation in the tissue from the hypothalamus and the pituitary (Table I) Even if the total uptake of fructose is less than that of glucose it is evident when regarding the values for the CO_2 and lactic acid formation that both are useful substrates for the different parts of the brain and the posterior pituitary

Beloff-Chain et al. (1955) incubated brain slices with ^{14}C-labelled fructose and found the same general metabolic pattern as when ^{14}C glucose was used. However they found the overall metabolism was lower for fructose than for glucose. Further t has been demonstrated in intact animals that there is a limited penetration of fructose into cerebral tissue (Stone 1938, Klein and Olsen 1947) After parenteral administration of ^{14}C fructose the uptake in different parts of the brain was lower than for glucose (Larsson and Lefkovsky in press) The uptake of labelled fructose as well as of glucose could, however be increased by the deprivation of food. In the present experiments the conversion of labelled fructose into CO_2 and lactic acid was considerably lower than when glucose was used. Concerning the CO_2 this was particularly pronounced in the samples from the hypothalamus and from the anterior pituitary As mentioned before the values for oxygen consumption and the $^{14}CO_2$ production after incubation with labelled fructose indicated a relative increase in the utilization of endogenous oxidisable substances. Further in comparison with the present results demonstrating a lower ^{14}C lactic acid formation with fructose it has been found that in cerebral tissue fructose and

mannose can yield lactate as rapidly as can glucose but that higher concentrations are required (GEIGER 1940 MEYERHOF and WILSON 1948)

BELOFF-CHAIN *et al.* (1955) found that approximately 18 % of the total fructose uptake by brain slices was accounted for by free unchanged fructose in the cells. In the present experiments the same observation was made. The corresponding figure for the cerebral cortex being about 14 % but in the anterior pituitary the amount of free unchanged fructose was considerably higher When calculating the absolute amounts of free unchanged fructose in the cells of the different parts studied there was no essential differences within the brain or between the two hypophyseal samples. It therefore seems reasonable to assume that it is not the transport mechanism for fructose suggested by BATTAGLIA and RANDLE (1959) which is responsible in this case for the differences observed in the fate of fructose and glucose in the various samples studied. In accordance with the findings by BELOFF-CHAIN *et al.* (1959) small amounts of ^{14}C glucose were formed from the labelled fructose in the medium. The explanation for the differences in fructose utilisation in the different samples studied is not fully established. It should be pointed out, however that evidence has been presented for the existence of a hexose monophosphate pathway for glucose oxidation in endocrine organs (FIELD *et al.* 1960) Further with the same experimental technique used in the present study these findings are supported (HELLMAN and LARSSON to be published) It also seems as certain parts of the hypothalamus should have a more active pentose shunt.

In brain slices, BELOFF-CHAIN *et al.* (1955) found that fructose qualitatively followed the same metabolic pattern as glucose. Thus, besides a generally lowered conversion of fructose into the different amino acids, no distribution differences were observed when compared to glucose. In the present experiments this observation was generally confirmed. Certain distribution differences existed however with regard to the amino acid formation. Thus, in the periventricular tissue and in the anterior pituitary the conversion of fructose into glutamic acid was proceeding at a comparatively lower rate when compared to the other amino acids found. As mentioned previously the amino acid formation in the anterior pituitary from the medium fructose was very low also for the other amino acids. As was the case for the CO formation in this part a corresponding increase in the endogenous formation of the amino acids could take place. Except for the anterior pituitary the decreased conversion of fructose into amino acids was less pronounced compared to the very marked decrease in CO_2 and lactic acid formation in most samples studied. It is therefore possible that the amino acid pool is very little affected by fructose as the substrate compared to glucose.

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References

ANDERSSON, B., S. LARSSON and F. POCCHIARI, Aspects on the glucose metabolism of the hypothalamus and the pituitary in goats. *Acta physiol. scand.* 1961 *51* 314—324.

BATTAGLIA, F. C. and P. J. RANDLE, Monosaccharide transport in rat diaphragm muscle, *Nature* (Lond.) 1959 *184* 1713—1714.

BELOFF-CHAIN A., R. CATANZARO, E. B. CHAIN, I. MASI and F. POCCHIARI, The fate of uniformly labelled ^{14}C fructose in the isolated rat diaphragm and rat brain and liver slices. *Ann. Acad. Sci. Fenn. A.* 1955 *60* 412—421

CHAIN, E. B., S. LARSSON and F. POCCHIARI, The fate of glucose in different parts of the rabbit brain. *Proc. Roy. Soc. B* 1960. *152* 283—289.

FIELD, J. B., P. JOHNSON, B. HERRING and A. N. WINEGRAD, Studies of pathway of glucose metabolism of endocrine tissues. *Endocrinology* 1960. *67* 801—806.

FRANK, M., E. B. CHAIN, F. POCCHIARI and C. ROSSI, An improved automatic scanner for the quantitative evaluation of bidimensional radiochromatograms. Selected Scientific Papers from the Istituto Superiore di Sanità, 1959 *2* 75—87

GEIGER, A., Glycolysis in cell-free extracts of the brain. *Biochem. J.* 1940. *34* 465—482.

HELLMAN, B. and S. LARSSON, To be published.

KLEIN, J. R. and N. S. OLSEN, Distribution of intravenously injected glutamate, lactate pyruvate, and succinate between blood and brain. *J. biol. Chem.* 1947 *167* 1—5.

LARSSON S. and S. LEPKOVSKY The uptake of ^{14}C-labelled glucose fructose and glycine in the brain of hungry and fed animals. *Amer. J. Physiol.* In press.

MCILWAIN, H., Glucose level, metabolism and response to electrical impulses in cerebral tissues from man and laboratory animals. *Biochem. J.* 1953 a. *55* 618—624.

MCILWAIN, H. Substances which support respiration and metabolic response to electrical impulses in human cerebral tissues. *J. Neurol. Neurosurg. Psychiat.* 1953 b. *16* 257—266.

MEYERHOF O. and J. R. WILSON, Glycolysis of brain preparations IV. *Arch. Biochem.* 1948. *17* 153—169.

STONE, W. E., The effect of anaesthetics and of convulsants on the lactic acid content of the brain. *Biochem. J.* 1938 *32* 1908—1917

VILLEE, C. A. and A. B. HASTINGS, Metabolism of C^{14}-labeled glucose by rat diaphragm *in vitro*. *J. biol. Chem.* 1949 *179* 673—687

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The Influence of Rapid Injection of Concentrated Potassium Chloride Solution on the Cardiovascular Functions of the Cat in Respiratory Disorders

By

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Abstract

Antila, L. and K. Hartiala. *The influence of rapid injection of concentrated potassium chloride solution on the cardiovascular functions of the cat in respiratory disorders.* Acta physiol. scand 1961 52 178—184 — The effect of concentrated potassium chloride solution on the cardiovascular functions of the cat has been studied under severe asphyctic hypoxic, and hypercapnic conditions The potassium concentrations varied between 330—670 meq/l and the total amount injected between 0.33—0.67 meqK or 0.15—0.35 meqK /kg b. w The blood oxygen saturation and pH as well as serum potassium content were analysed before the injection. The effect of the injection was followed by continuous electrocardiogram and aortic pressure recordings. The 30 min period of respiratory disturbance did not cause any uniform changes in the serum potassium content. The cardiovascular changes prior to the injection were also unremarkable The results indicate that the tolerance of the cat towards high potassium concentrations is mainly depressed by lack of oxygen whereas an excess in carbon dioxide does not appear to be so dangerous.

Respiratory disturbances have shown to cause at least in dogs changes in the distribution of potassium It has been found that potassium is mobilized from the cells during tissue anoxia (Dennis and Moore 1938 Mullins et al 1938) in respiratory acidosis (Scribner et al 1954 Burnell et al. 1956) and

during respiratory alkalosis (GERBISH *et al.* 1955 HICKAM *et al.* 1956) MULLIN *et al.* (1938) have claimed the serum potassium increase rather being due to the oxygen lack than the carbon dioxide excess. An increase in the potassium has been shown to take place in pure respiratory acidosis (SCRIBNER *et al.* 1954) In the present investigation studies have been made of the tolerance of the cat towards acute concentrated potassium injections during various respiratory disturbances.

Material and Methods

Cats of both sexes weighing 1.5—4.4 kg were used in the experiments. The material was divided into two groups. In the first one the potassium tolerance was studied in the same cats subjected to different disturbances. In the other group each cat received th same potassium injection under certain disturbance condition.

The animals were anesthetized with 0.07 g/kg of nembutal given intraperitoneally. The tracheal cannula was connected into a 250 l plastic bag containing different gas mixtures. These were in the asphyxia experiments 7 % O_2, 13 % CO_2 and 80 % N in the anoxia experiments 7—10 % O_2, 0—3 % CO_2 and 7—90 % N and in the hypercapnia experiments 20—80 % O_2, 13—20 % CO and 0—67 % N. The expiration was conducted by means of valves into the room air. It was also possible to allow the cat breath through the valves regular room air.

The II lead was used in the ECG registration. The pressure recordings were mad from the femoral artery through catheter which was brought up to the region of the thoracic aorta. Blood samples were also taken through this catheter. Another catheter was introduced through the femoral vein 15 cm into the inferior caval vein. The potassium was administered through this catheter.

From the blood samples the hemoglobin, oxygen saturation, potassium concentration and the arterial pH and also in some case the blood oxygen and carbon dioxide contents were determined.

Results

General. Preliminary experiments were performed on 3 cats which were exposed to asphyxia and hypoxia over a period of 60 min by using different gas mixtures. By reducing the oxygen content in these mixtures to 7 % a marked drop down to 75—52 per cent was achieved in the oxygen saturation. The results were similar after 30 and 60 min with regard to the cardiovascular changes and laboratory analyses and the cats tolerated well the condition. Since it has been shown in dogs that oxygen is harmful in respiratory acidosis (MILLER *et al.* 1952 SCRIBNER *et al.* 1954) pure oxygen was given to these cats after the exposure to asphyxia. No harmful side-effects could be noted.

Laboratory analyses. The low oxygen saturation values in hypoxia and asphyxia in Fig. 1 indicate that the condition has really been grave. No consistent changes in the serum potassium concentrations were found since in some cases there was a rise whereas in some cases even a drop was present and there were values below the control values particularly in connection with hypoxia. In some cases potassium had been administered before the

Fig 1 Laboratory data on cats in atmosphere and in different respiratory disorders.

atmosphere, asphyxia, ● hypoxia, hypercapnia.

appearance of serum potassium concentrations higher than control values. The blood pH analyses showed that the animals were in deep acidosis both during asphyxia and hypercapnia. This acidosis has been of the respiratory type as indicated by the occasional blood carbon dioxide determinations and as such what could be expected under these experimental conditions. In few cases the low pH values at the beginning of the experiment under normal atmospheric conditions may be interpreted as a result of hypoventilation caused by the anesthesia.

The general cardiovasculatory observations in various respiratory disturbances showed only minor changes. The most consistent change was the rise in the pulse pressure. It had dropped only in two cases and remained unchanged in another two. The systolic pressure frequently increased, and in all cases in hypercapnia.

The changes in the ECG were of no significance. Such were more frequent in the first experimental series, obviously because in these potassium was administered between the registrations whereas in the latter series the animals were only exposed for respiratory disturbances before the ECG control.

The effect of potassium injections on the cardiovasculatory functions. Potassium, when administered rapidly and in concentrated solutions, caused similar

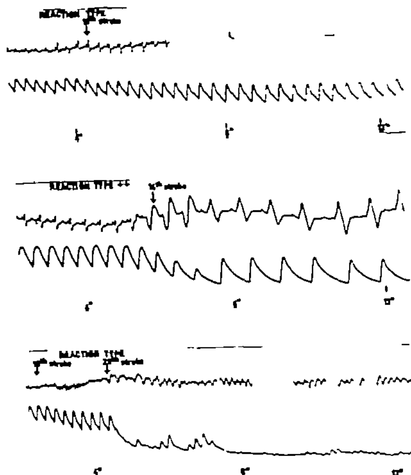


Fig. 2. Types of reaction to acute concentrated potassium injection. Type + = mild potassium effect, Type ++ = potassium block, Type +++ = potassium fibrillation.

changes as those described previously by others (e g Wiggers et al. 1930 Nahum and Hoff 1939) These changes have been characterized by lowering of the ST-interval, disappearance or negativity of the T wave (Fig 2. Reaction type +) The condition improved in few seconds or developed into a block (Fig 2. Reaction type ++) or was followed by potassium fibrillation (Fig 2 Reaction type +++) The block could improve spontaneously in few seconds but the fibrillation could persist over a period of more than two minutes and then spontaneously improve. A high positive T wave appeared 20—30 sec after the potassium injection or about 10—15 sec after the cessation of the fibrillation.

Table I Reaction to potassium injections in atmosphere and in different respiratory disorders

Degree of reaction	Atmosphere Cat no.	Number of injections	Asphyxia Cat no.	Number of injections	Hypoxia Cat no.	Number of injections	Hypercapnia Cat no.	Number of injections
+	4 9	2 2	9	2	9	2	22	3
++	5, 6	2 2	5	2	—	—	10	2
+++	8, 10 11 12, 13	8, 2 1 3, 3	4 6 7 11 14 15	1 1 2 2, 1 1	18 19* 20, 21	1 1 1, 3	11 16 17 23	3, 1 3 1

+ = mild potassium effect, ++ = potassium block, +++ = Potassium fibrillation as depicted in fig 2. Cat marked with asterisk died

The slowing of the pulse rate and the block provoked by potassium was accompanied by a decrease in the blood pressure but effective pulsations were present throughout this period (Fig 2) During the fibrillation period on the other hand no effective pulsations were present and the electric activity of regular appearance which may appear at the later phases was not associated with mechanical efficiency and a rise in the pressure.

The effect of potassium injection in our series is presented in Table II The results of the first experimental trial illustrate the effect in the cases when potassium was administered during the disorders produced at 30 min intervals. The order of the injections was varied in order to eliminate the cumulative effect of potassium on the interpretation of the results. The potassium injections were in each condition given in 5 min intervals and the intensity of the reaction is marked in the table.

In the first series 2 or more consecutive potassium injections were given. The effect of the injection is marked with + if the changes in the ECG were moderate ++ if they were followed by a block and +++ if a fibrillation was produced (vide Fig 2) In the latter experimental trials attempts were made to produce such fibrillation and the number of injections necessary for this was reported.

The results in the table indicate that under normal atmospheric conditions the cats have tolerated the administered potassium distinctly better than in the disturbancy cases. Cats no. 4 5 9 10 and 11 received injections first in asphyxia, hypoxia or hypercapnia and tolerated the injections in atmosphere well. When the injection was given in atmosphere first as in cat 6, the cat reacted very seriously when the injection was repeated in asphyxia. In hypercapnia the tolerance appeared better than in asphyxia or hypoxia. Respiratory acidosis appeared therefore not to be a determining factor for the lowered potassium tolerance but that the role of hypoxia seems to be more decisive.

Discussion

The toxic effect of potassium in cats appears to be increased during oxygen lack. The results obtained in the experiments have somewhat varied in different animals but the observations made in different conditions have shown that the lack of oxygen has more importance than the excess of carbon dioxide (e g MATHISON 1910, GREENE and GILBERT 1922)

Permanent fibrillation was produced by the first potassium injection only in one cat during hypercapnia although the degree of acidosis was as great as in the asphyxia experiments.

The total amounts of administered potassium were small and obviously the applied concentrations have caused the changes. Even after repeated injections the serum potassium concentrations were not high. Likewise high potassium concentrations were not found as a result of respiratory acidosis even though occasional elevations were present. The response of the cats towards asphyxia seems thus to differ somewhat from that of the dog. Oxygen administration did not cause any cardiovascular changes in the cats. In dog the same has even caused a cardiac arrest (MILLER et al. 1952) W thin normal pH conditions the dog has not tolerated potassium as well as during respiratory acidosis (SCHROEDER et al. 1954) In the cat the matter is reversed.

These experiments do not yet indicate the cause of the diminished potassium tolerance. Several factors may be responsible for it. The applied disturbancy of a duration of half of an hour has not been able to cause a consistent rise n the serum potassium concentrations. There may still be a tendency for an escape of potassium from the cells perhaps as a result of the lack of energy due to hypoxia. Also increased adrenal cortical activity at least in asphyxia may be responsible for the fact that the animals have not tolerated the applied exogenous potassium during respiratory disturbancy as well as in a normal atmosphere. Also a direct cardiac effect of potassium should be considered. The use of radioactive potassium — though — has not been able to clarify why some animals have recovered more rapidly from the potassium influence than others in studies by KAPLAN and FISHER (1959) Still the question is a matter of interest since potassium is used as a cardioplegic substance in open heart surgery (MELROSE et al 1955) Obviously its use is accompanied by difficulties. The possible cause of them, we hope, have been partially elucidated by the present investigation.

References

BURNELL, J M M F VILLAMIL, B. T UYENO and B. H. SCRIBNER, The effect in humans of extracellular pH change on the relationship between serum potassium concentration and intracellular potassium. J clin. Invest. 1956. 35 935—939

DUNCAN, J and R. M. MOORE, Potassium changes in functioning heart under conditions of ischemia and of congestion. Amer J Physiol 1938. 123 443—447

Giebisch, G., L. Berger and R. F. Pitts, The extrarenal response to acute acid-base disturbances of respiratory origin. *J. clin. Invest.* 1955. *34*: 231—245.

Greene, C. W. and N. C. Gilbert. Studies on the responses of the circulation to low oxygen tensions. *Amer. J. Physiol.* 1922. *60*: 155—192.

Hickam, J. B., W. P. Wilson and R. Frayser, Observations on the early elevation of serum potassium during respiratory alkalosis. *J. clin. Invest.* 1956. *35*: 601—606.

Kaplan, A. and B. Fisher, Study of the cardioplegic action of potassium ion during normothermia and hypothermia. *Ann. Surg.* 1959. *150*: 833—840.

Mathison, G. C., The cause of the heart block occurring during asphyxia. *Heart* 1910. *2*: 54—73.

Melrose, D. G., B. Dreyer, H. H. Bentall and J. B. E. Baker, Elective cardiac arrest. *Lancet* 1955. *II*: 21—22.

Miller, F. A., E. B. Brown, J. J. Buckley, F. H. van Bergen and R. L. Varco, Respiratory acidosis: Its relationship to cardiac function and other physiologic mechanisms. *Surgery* 193_. *3_*: 171—183.

Mullin, F. J., J. J. Dennis and D. B. Calvin, Blood potassium in tetany and asphyxia of dogs. *Amer. J. Physiol.* 1938. *1_4*: 192—201.

Nahum, L. H. and H. E. Hoff. Observations on potassium fibrillation. *J. Pharmacol.* 1939. *65*: 322—331.

Richards, J. B. and S. S. Stein. Effect of CO exposure and respiratory acidosis on adrenal 17-hydroxycorticosteroid secretion in anesthetized dogs. *Amer. J. Physiol.* 1957. *188*: 1—6.

Scribner, B. H., G. M. Burnell, K. Fremont-Smith and J. M. Burnell, Potassium intoxication during and immediately following respiratory acidosis. *J. clin. Invest.* 1954. *33*: 965—966.

Wiggers, C. J., H. Theisen and H. D. B. Shaw. Studies on ventricular fibrillation produced by electric shock III. The Action of antagonistic salts. *Amer. J. Physiol.* 1930. *93*: 197—212.

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The Effect of Histamine on the Uptake of ^{35}S by the Duodenal Mucosa of Guinea Pigs

By

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Abstract

Ball, P. I. Härkönen and K. Hartiala. *The effect of histamine on the uptake of ^{35}S by the duodenal mucosa of guinea pigs.* Acta physiol. scand. 1961. 52. 185—187. — The purpose of the work was to find out to what degree the effect of promoting the uptake of ^{35}S was dependent upon free access of acid to the duodenal lumen. The pylorus was ligated in 32 young male guinea pigs. Histamine in a dose of 3 mg/kg body weight was administered subcutaneously as the ^{35}S sulphate given immediately after the operation. The animals were sacrified 24 hours later. It was found that the uptake of ^{35}S sulphate was increased both in the duodenal mucosa and in the underlying "stroma". The results would indicate that in the guinea pig histamine stimulates the synthesis of mucus in the duodenum also under conditions where the effect of acid has been eliminated.

Brunner's glands secrete at a faster rate when acid is introduced into the duodenum (Florey, Wright and Jennings 1941). This response is at least partly mediated by the local release of a humoral agent (Sonnenschein, Grossman and Ivy 1947). Goblet cells empty themselves of mucus in response to local applications of acid or irritants, the factors which affect the rate at which they secrete are otherwise uncertain.

Härkönen (1961) showed that in guinea pigs a single small dose of histamine would cause an increase in the quantity of sulphate released by acid hydrolysis from the deepest layers of the mucosa and the underlying connective tissue of the distal duodenum. It was concluded that the increase was dependent upon synthetic activity in mesenchyme. The increase could still be demonstrated after acid had been excluded from the duodenum by ligature of the pylorus.

Table 1. The radioactivity of the tissues of the duodenal wall of guinea pigs 24 hours after injection of $Na_2{}^{35}SO_4$. Pyloric ligation was performed on all the animals. The treated group was also given histamine in beeswax 5 mg/kg body weight.

Sample	Control group		Histamine treated group	
Counts/mg dry weight/min.	mean	S. E. M.	mean	S. E. M.
Duodenal mucosa	13.5	1.4	19.0	1.3
Duodenal stroma	5.2	0.6	9.9	0.7

In the superficial layers of the distal duodenal mucosa of intact animals the uptake of ^{35}S labelled sulphate was augmented by histamine although histamine caused no perceptible change in its sulphate content as determined chemically. It was suggested that the uptake of labelled sulphate under these circumstances reflected the secretory activity of mucous cells.

The purpose of the work to be described in this paper was to find out to what degree the effect of histamine in promoting the uptake of ^{35}S labelled sulphate was dependent upon free access of acid to the duodenal lumen.

Material and methods

Thirty-two young male guinea pigs were used. The pylorus was tied under ether anesthesia. Immediately after operation ^{35}S was given subcutaneously as $Na_2{}^{35}SO_4$ (obtained from Radiochemical Centre, Amersham, England) in a dose of 400 μc/kg body weight, with 16 mg of Na_2SO_4 as carrier. The animals were then separated into two equal groups. One group served as controls. The animals in the other group were given histamine in beeswax subcutaneously in a dose of 5 mg/kg body weight in antihistamine cover (Antistine Ciba 20 mg/kg body weight subcutaneously). It was stated in preliminary experiments that antihistamine used had no effect on the sulphate content or uptake of *the radiosulphate in the gastroduodenal tract of guinea pigs.*

The animals were killed 24 hours after operation. The duodenum was prepared by the method described by HÄKKINEN (1961). In brief, the mucosa was scraped off from the underlying tissue of a standard length of duodenal wall, distal to the ampulla of Vater, *in an area where the guinea pigs* BRUNNER *glands are diffuse and relatively* scanty. Microscopical examination showed that the mucosa which was scraped away contained the villi and the greater part of the crypts of LIEBERKÜHN, and that the stroma remaining consisted of some rests of the crypts, the muscularis mucosae containing BRUNNER*'s glands and the muscle and serosa.* The dry weight of both mucosa and stroma were measured and the tissues were separately homogenized, hydrolysed and prepared for counting of radioactivity by a plate technique as previously described (HÄKKINEN 1961).

Results

The results are expressed as counts per minute/mg dry weight of tissue, and are shown in Table 1.

The difference between the values for the two groups is significant for the mucosa ($0.01 > P > 0.001$) and highly significant for the stroma ($P < 0.001$).

Discussion

ODEBLAD and BOSTRÖM (1952) have shown that such radioactive sulphate as is fixed in the tissues is almost all incorporated into sulphomucopolysaccharides, and HÄKKINEN (1961) confirmed that in the duodenal wall nearly all suphate, whether determined chemically or in terms of radioactivity 24 hours after injection of 35S was contained in substances of high molecular weight. If on the other hand the radioactivity of the duodenal secretions is measured 4 hours after injection of 35S sulphate or later only some 69 % of the activity is found to be contained in high molecular organic compounds, that is in sulphomucopolysaccharides. The rest is present as inorganic sulphate or conjugated sulphate (KENT *et al.* 1956) This difference between the tissue and its secretions indicates that the duodenal mucous cells do not store inorganic or conjugated sulphate the rate of transit of these substances through the duodenal wall is rapid, and the quantity present at any one time is accordingly very small.

The activity 24 hours after 35S was given was the same in the untreated animals in this study as that recorded by HÄKKINEN (1961) in intact animals. The operation of pyloric ligation thus appears to exert no net effect. In the operated animals histamine caused an increase in activity of about 90 % in the deep layers of the duodenum. This change in the stroma as well as the increase in the sulphate content as measured chemically may well correspond to the suggested action of histamine on the synthetic activity of connective tissue. The activity increased about 40 % in the more superficial layers of the mucosa, whereas increase was about sixfold when there was normal continuity between the stomach and duodenum (HÄKKINEN 1961) If one accepts that the rate of uptake of 35S sulphate into mucosal sulphomucopolysaccharides is proportional to the rate of synthesis of mucus in the gland cells, it is evident that histamine still exerted a stimulating effect upon this synthesis when acid had been excluded from the duodenum. Either the effect was directly produced by histamine, or some additional path way of stimulation exists whose receptors lie outside the duodenum.

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References

FLOREY H. W R. D. WRIGHT and M. A. JENNINGS, The secretions of the intestine. *Physiol Rev* 1941 *21* 36.

HÄKKINEN, I. P T Histamine induced changes in the sulphate metabolism of the duodenum and prepyloric region of the stomach An experimental study with special reference to ulcer formation *Acta physiol. scand* 1961. *51* Suppl. 177

KENT P W M. W WHITEHOUSE, M A. JENNINGS and H. W FLOREY Observations on the incorporation of 35S in duodenal mucosubstances. *Quart. J exp. Physiol.* 1956. *41* 230—246.

ODEBLAD, E. and H. BOSTRÖM, An autoradiographic study of the incorporation of 35S labelled sodium sulfate in different organs of adult rats and rabbits. *Acta path. microbiol. scand.* 1952, *31* 339—344

SONNENSCHEIN, R. R., M. I. GROSSMAN and A. C. IVY Humoral regulation of Brunner's glands. *Acta med. scand.* 1947 Suppl. 196. 296.

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Gastro-intestinal Excretion of Dextran-C^{14}

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Abstract

Åberg, B., W. L. Bloom and E. Hansson. *Gastrointestinal excretion of dextran-C^{14}*. Acta physiol. scand. 1961. 52. 188—194. — Cats were given dextran-C^{14} intravenously and the gastric juice as well as intestinal juices from intestinal segments and bile was collected during the first 6 hours after administration. The juices were investigated for radioactivity. It was found that there is small excretion of radioactivity via the stomach and the liver. The intestinal juices however and chiefly those from the cranial parts of the intestine contained larger amounts of radioactivity which was precipitable with ethanol and represented dextran. The earlier literature on dextran metabolism is reviewed. The parallelism between the dextran and the albumin elimination via the gastrointestinal tract is pointed out.

Partly hydrolyzed, bacterial dextran introduced as a plasma substitute by Grönwall and Ingelman in 1944 has been extensively tried clinically during the last 15 years. The metabolism has been studied by numerous authors and their results were recently reviewed by e. g. Squire et al. (1955) and Grönwall (1957).

It has been demonstrated that starch-splitting enzymes do not break down dextran (Grönwall and Ingelman 1945, Ingelman 1947, Bull et al. 1949). Persson (1952) found evidences that dextran is altered in the tissues in a way which makes it stainable with leuco-fuchsin without previous treatment with periodic acid. This author presumed that the change was due to oxidation of dextran. Schmitz (1951) found that dextran was broken down by horse leucocytes in vitro as judged by viscosimetric determinations. Gray, Siteri and Pulaski (1951) presented evidences that dextran is metabolized to glu-

cose in the phlorizinized dog Uptake and storage of dextran in various organs *i. e.* liver spleen, kidney was reported by several investigators *e. g.* BULL *et al.* (1949) ENGSTRAND and ÅBERG (1950) FRIBERG GRAF and ÅBERG (1951 1953) SWEDIN and ÅBERG (1952)

Gastro-intestinal excretion of dextran was found by ENGSTRAND and ÅBERG (1950) in rats and rabbits and by TROELL and ÅBERG (1953) in man. Contradictory results were reported by several investigators (cit. GRÖNWALL 1957) The chemical methods employed for determination of dextran in biological fluids, have been and are very unspecific. Thus reactions with carbazol (ÅBERG 1952) or anthrone (DURHAM *et al.* 1950) will give results greatly influenced by all carbohydrates able to form furfural in the presence of conc. sulfuric acid. Turbidimetric determination after precipitation with ethanol as introduced by JACOBSSON and HANSEN (1952) gives fairly correct values in blood serum or plasma but cannot be used on gastro-intestinal contents. The nonspecificity of the chemical reaction might have caused the differences in opinion as to the gastrointestinal secretion of dextran.

BLOOM and WILHELMI (1952) presented evidence that there is an enzyme in the duodenal contents able to break down dextran rapidly and ÅBERG (1953) showed that human faeces breaks down dextran to low molecular fatty acids.

Synthesis of dextran-C¹⁴ made it possible to study the metabolism of dextran further CARGILL and BRUNER (1951) showed that dextran-C¹⁴ was metabolized in mice — the expired air of the animals contained $C^{14}O_2$ and the urine contained C^{14}-carbonate. In man, HELLMAN (1951) studied the metabolism of dextran-C¹⁴ (Commercial Solvents-Argonne National Laboratory Lot 21—2—Ci—L—D) and found that in 10 days 64 per cent of the radioactivity was excreted in the urine and 26 per cent as $C^{14}O_2$ in the expired air About 2 per cent appeared in the faeces. HELLMAN did not study the gastrointestinal contents of his patient.

Injection of tritium-labelled dextran into mice showed that there was a clear gastro-intestinal excretion of the tritium (HANNGREN *et al.* 1959) Although it was possible to precipitate the activity with ethanol from the urine which showed that the tritium had stayed on the dextran molecules it was deemed of interest to study the excretion further A series of experiments with C^{14}-labelled dextran were performed on cats as one of us (W. L. B.) had dextran-C¹⁴ of the same brand as that used by HELLMAN (1951)

Material and Methods

C¹⁴-Dextran

Generally C^{14}-labelled, partly hydrolysed dextran (Commercial Solvents-Argonne National Laboratory Lot 21—2—Ci—L—D) as 6 per cent sterile solution in saline was used. The specific activity was 6 µC of C^{14} per g of dextran. Physical-chemical data

Table 1. Excretion data for dextran-C (CSC-4 gamma Lot no. 21 2-Cl L-D)

Cat no.	Weight kg	ml of C14 dextran	Cpm of C-14	Time (hrs after inj.)	Excretion cpm in Urine	Bile	Stomach
1	3.4	25	7.5 10	6	3.6 10⁴		1.08 10
	2.5	19	5.7 10⁴	6	2.3 10	1.67 10	
3	3.9	28	8.4 10	6	4.3 10⁴		
4	3.9	22	6.6 10⁴	6	3.5 10⁴	1.31 10	
5	3.4	25	7.5 10	2	2.8 10		
6	2.3	18	5.4 10	6	2.9 10⁴		
7	2.9	28	8.4 10	6	4.3 10⁴		5.62 × 10⁴

for this lot of dextran have been presented by WEINBERG and ISBELL (1951) — light scattering revealed mean molecular weight of 66 000

Injection. The dextran-C was given in the femoral vein

Urine samples. a polyethylene cannula was introduced in the urethra int the urinary bladder and urine samples were taken at 1 hour hours, 4 hours and 6 hours after the injection of dextran.

Blood samples. permanent cannula was introduced into the carotid artery and blood samples taken 5 min, 15 min, 1 hour 2 hours, 4 hours and 6 hours after injection of dextran

Bile A polythene tube was introduced into the hepatic duct after ligation of the cystic duct. The bile samples were taken t 1 hour 2 hours, 4 hours and 6 hours after the injection of dextran.

Perfusion of the intestine Cats starved 24 hours were anesthetized with chloralose urethane. An incision was made in the abdomen od section was removed about 15 cm below the stomach and in some experiments 15 cm abov the caecum. An incision was made in the gut and two rubber balloons was introduced into the intestine according to th arrangement described by ARMSTRONG, MARGEN and TARVER (1960) With this arra gement of balloons about 70 cm long section of the intestine is isolated. The intestine was washed by flowing 2 per cent dextran solution through a polyvinyl tube and taking out the perfusion fluid via another The samples were taken t 1 hour 2 hours, 4 hours and 6 hours after the injection of dextran. The washing solution was tested for blood by the benzidine method

Stomach content The oesophagus was ligated just bov the cardia. A small incision was made in the duodenum od a glass cannula introduced into the stomach. The stomach secretion was timulated by a subcutaneous injection of 0.1 mg histamine hydrochloride per 10 kg body weight every hour The stomach content was removed by continuous suction and washing of the stomach with physiological saline. The samples were taken t 1 hour 2 hours, 4 hours and 6 hours after injection of radioactive dextran.

Radioact (assay The radioacti ity in the samples of urine blood, bile tomach and intestinal contents was estimated in gas flow counter after plating an amount of the samples on aluminium dishes in infinitely thin layers according to RYDBERG (1958).

Assay for radioactivity in the ethanol precipitable fraction of the intestine contents and the stomach contents were carried out as follows to sample of the washing solution an equal amount of an ice-cold 20 per cent trichloroacetic acid solution was added

Excretion as cpm in		Excretion as per cent of dose in				
Ileum	Jejunum	Urine	Bile	Stomach	Ileum	Jejunum
x	x	48.0	x	0.14	x	
x	x	40.4	0.3	x	x	
x	1.27 10	51.2	x	x	x	1.3
x		53.0	0.2	x		x
7.8 10[4]	x	37.3	x		0.1	x
3.23 x 10[4]		53.7	x		0.6	x
	1.56 10	51.2	x	0.7	x	1.6

in order to precipitate the protein and followed by centrifugation t 3,000 *g* for 10 minutes. The supernatant was taken and ethanol was added to concentration of 60 % following centrifugation of 3,000 *g* for 10 minutes and the sediment was washed three times in ethanol. Thereafter the sediment was dissolved in water and plated on aluminium dishes in infinitely thin layers. The radioactivity was estimated in a gas-flow counter.

Results and Discussion

The individual results are summarized in Table I. Obviously there is an excretion of radioactivity in the stomach, the bile and the intestines. In the urine about 50 per cent of the dose was excreted within 6 hours after injection (Fig. 1). In Fig. 2 the blood plasma content of radioactivity — representing circulating dextran-C[14] is given for 3 animals.



Fig. 1

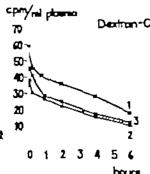


Fig. 2

Fig. 1. Cumulative elimination of radioactive material in urine of cat no. 3 following intravenous injection of dextran-C[14]

Fig. 2. Variation with time in blood plasma radioactivity following intravenous injection of dextran-C[14] in cat no. 1, 2 and 3.

Table II Percentage of radioactivity excreted precipitable with ethanol (60 per cent final concentration)

Cat no.	Juice/organ	Per cent precipitated
1	gastric	24.2
2	bile	18.9
3	jejunum	39.8
4	bile	72.2
5	ileum	82.9
6	ileum	59.0
7	jejunum	28.3
7	gastric	24.7

In the gastro-intestinal tract, the bile secretion of radioactivity is fairly small, amounting to some 0.2 per cent of the dose. The gastric secretion of radioactivity is also fairly small whereas the intestinal secretion is considerable, amounting to a maximum of 1.6 per cent of the dose in cat no. 7 in a 20 cm part of jejunum after 6 hours.

Of interest is the percentage of the radioactivity excreted which is precipitable with ethanol. Table II shows that this amount varies considerably. The variation at a final ethanol concentration of 60 per cent, is probably due to the bacterial and enzymatic decomposition of dextran which occurs in the juices during the collection time.

The urinary excretion (Fig. 1) and the plasma contents of radioactivity (Fig. 2) follow those earlier reported for this lot of dextran in e. g. man (HELLMAN 1951). The bile excretion is shown in Fig. 3 and is fairly small — as is seen from the figures given in Table I the bile excretion has the same order of magnitude as the gastric excretion.

The experiments reveal that intestinal excretion plays a major role in the elimination of dextran intravenously administered to cats. Some excretion also takes place via the bile and the stomach but these routes seem to play a minor role during the first 6 hours after administration.

It is not possible to make any quantitative conclusions from the experiments performed but it is possible to make a rough estimation of the actual amount of radioactivity which appears in the whole intestine during the first six hours after injection of dextran-C[14]. In cat no. 3 there was an excretion of 1.5 per cent of the dose in the 20 cm of intestine ligated. Assuming that the excretion is roughly the same in all parts of the intestine and that it has a total length of 1.4 m, about 10 per cent of the injected dose should be eliminated in this way.

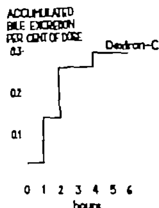


Fig. 3. Cumulative elimination of radioactive material in bile of cat. no. 2 following intravenous injection of dextran-C^{14}

It can be concluded from these experiments that the intestinal elimination of dextran plays a major role in the cat. It has to be borne in mind, however that the caudal part of the intestine eliminates far less dextran than the cranial part (vide Table I cats no. 5 and 6) and thus the actual amount of dextran excreted intestinally during the first 6 hours after i. v. administration must be far lower than 10 per cent. Even so intestinal excretion of dextran as well as urinary must be considered the natural way of elimination of dextran. It is possible that serum albumin and perhaps other plasma proteins like dextran are excreted into the gastrointestinal tract where they are hydrolysed, since it has been showed that albumin-I^{131} is excreted in the stomach and small intestine by man and animals (Birke *et al.* 1958, 1959 Armstrong, Margen and Tarver 1960) Ullberg *et al.* (1960) showed autoradiographically that albumin-I^{131} is gastrointestinally excreted also in the cat. In exudative gastro-enteropathy" a gastro-intestinal excretion of albumin has been presumed likely (Kimbel *et al.* 1956, Schwartz and Jarnum 1959 cit. Ullberg *et al* 1960) These results suggest that gastrointestinal excretion of macro molecules may represent a hitherto unemphasized pathway of metabolism.

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References

Åberg, B. Some experiments on partly hydrolysed, bacterial dextran. Thesis, Karolinska institutet, Stockholm 1952.

Åberg, B., Breakdown of dextran by human faeces. *Scand. J. clin. Lab. Invest.* 1953. *5.* 37—38.

Armstrong, F. B., S. Margen and H. Tarver, Plasma Protein VII. Site of Degradation of Serum Albumin. *Proc. Soc. exp. Biol.* (*N. Y.*) 1960. *103.* 592—596.

Birke, G., S. O. Liljedahl and L.-O. Plantin, Albuminomsättningen vid brännskador studerad medelst 131 J-märkt albumin. *Nord. Med.* 1958. *60.* 963.

Birke, G., S. O. Liljedahl, L.-O Plantin and J. Wetterfors, Ventrikelns roll i albuminmetabolismen. *Nord. Med.* 1959. *62.* 1741—1744.

BLOOM, W. L. and A. WILHELMI cit. BLOOM, W. L., G. ADROUNEY and A. WILHELMI, Progr. Rep. April 14, 1948 to Subcommittee on Shock. A Dextran-splitting enzyme in the animal system. (USA.)

BULL, J. P., C. RICKETTS, J. R. SQUIRE, W. D. A. MAYCOCK, S. J. L. SPOONER, P. L. MOLLISON and J. C. S. PATERSON, Dextran as plasma substitute. Lancet 1949. 256. 6541.

CARGILL, W. H. and H. D. BRUNER, Drug Trade News 1951. 26. 46.

DURHAM, F. W., W. L. BLOOM, G. T. LEWIS and E. E. MANDEL, Public Health Report 1950. 65. 670 cit. Chem. Abstr. 1950. 44. 7919 b.

ENGSTRAND, L. and B. ÅBERG, Excretion of intravenously administered dextran. Lancet 1950. 1071—1072.

FRIBERG, U., W. GRAF and B. ÅBERG, On the histochemistry of partly hydrolysed, bacterial dextran. Scand. J. clin. Lab. Invest. 1951. 3. 221—227.

FRIBERG, U., W. GRAF and B. ÅBERG, Effects of prolonged dextran administration to rabbits. Acta pharmacol. (Kbh.) 1953. 9. 220—234.

GRAY, I., P. K. SOTTERI and E. J. PULASKI, Metabolism of plasma substitute. I. Dextran (Macrodex). Proc. Soc. exp. Biol. (N. Y.) 1951. 77. 626.

GRÖNWALL, A., Dextran and its use in colloidal infusion solutions. Almqvist & Wiksell. Uppsala 1957.

GRÖNWALL, A. and B. INGELMAN, Några nya kolloidlösningar för infusionsändamål. Nord. Med. 1944. 21. 247—255.

GRÖNWALL, A. and B. INGELMAN, Untersuchungen über Dextran und sein Verhalten bei parenteraler Zufuhr II. Acta physiol. scand. 1945. 9. 1—27. cit. 6.

HANSSON, Å., E. HANSSON, S. ULLBERG and B. ÅBERG, Fate of injected dextran labelled with tritium in mice. Nature (Lond.) 1959. 184. 373—374.

HELLMAN, L., Prel. Rep. on the metabolism of C¹⁴-labelled dextran and PVP. Subcommittee on Shock. October 1951. (USA.)

INGELMAN, B., Dextran and its use as plasma substitute. Acta chem. scand. 1947. 1. 731—738. cit. p. 734.

PERSSON, B., Histochemical studies on parenterally administered dextran in rabbits. I. Demonstration of dextran in tissue sections. II. On the accumulation of dextran within the kidney, liver, leukocytes and reticulo-endothelial system. Acta Soc. Med. upsalien. 1952. 57. 411—432.

RYDBERG, J., Determination of the absolute activity of solid tritium samples. Acta chem. scand. 1958. 12. 399—407.

SCHMITZ, H., Über das Schicksal des Macrodex im Organismus. Klin. Wschr. 1951. 29—30. 424.

SQUIRE, J. R., J. P. BULL, W. D. A. MAYCOCK and C. R. RICKETTS, Dextran, its properties and use in medicine. Blackwell, Oxford 1955.

SWEDIN, B. and B. ÅBERG, On dextran in spleen, liver and muscle after intravenous injection into rabbits. Scand. J. Clin. & Lab. Invest. 1952. 4. 68—70.

TURELL, L. and B. ÅBERG, The carbohydrate content of the gastric juice after intravenous administration of partly hydrolysed, bacterial dextran to hospital cases. Scand. J. clin. Lab. Invest. 1952. 4. 355—358.

ULLBERG, S., G. BIRKE, B. EWALDSSON, E. HANSSON, S. O. LILJEDAHL, L.-O. PLANTIN and J. WETTERFORS, The role of gastrointestinal tract in the elimination of serum albumin. Acta med. scand. 1960. 167. 421—425.

WEISSBERG, S. G. and H. S. ISBELL, Molecular properties of plasma substitutes. National Bureau of Standards, Progr. Rep. to the Surgeon General June 9 to Sept. 26, 1951.

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Methods for Perfusing the Giant Axon of Loligo Pealii

By

T. OIKAWA, C. S. SPYROPOULOS, I. TASAKI and T. TEORELL

It was possible to perfuse the interior of the giant axon of *Loligo pealii* or *forbesi*, [C. S. Spyropoulos, I. Tasaki and T. Teorell, National Institutes of Health Annual Report, NINDB, 1959; Baker P. F., A. L. Hodgkin and T. D. Shaw Preprint of Proc. Physiol. Soc. (1961) also cited in preprint, Baker P. F. and T. D. Shaw (1961) Plymouth Marine Biological Laboratory report for 1960—1961 (in press)] with an artificial solution and maintain the resting potential and the ability of the axon to produce normal action potentials. The technique employed (Diagram A) in the first series of experiments was as follows. Using an axon of *Loligo pealii* about 25—35 mm in length, a column of axoplasm was removed by introducing a capillary (300 μ in diameter). Subsequently each end of the axon was cannulated using glass pipettes (approx. 200—300 μ in diameter) and fine nylon thread. The perfusing fluid was introduced through one cannula and drained through the other. The rate of perfusion varied; in most experiments it was of the order of 0.01 ml/sec. The perfusion rate was usually but not always controlled with the Phipps and Bird Syringe-Driver. This rapid rate effected a rapid equilibration of the diffusible components of the intracellular space. A pair of silver wires was introduced through the cannula for stimulation and recording (under space clamp conditions). This electrode assembly facilitated drainage by guiding the perfusate from the ejecting to the withdrawing cannula. In another series of experiments, this was accomplished by means of a calomel micropipette which was also used as a potential recording electrode. In later experiments, simpler methods were used and longer axons (35—50 mm). In the method illustrated by diagram B a long glass capillary (350 μ in diameter) with a long slit (20 mm long and 100 μ in width) on one side was employed.

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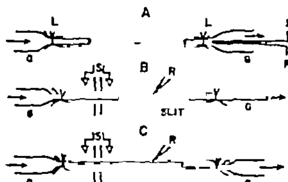


Fig. 1. Illustration of various methods of perfusing isolated squid giant axons. — A: Two separate glass cannulae (G) are tied with nylon thread (L) to the ends of the axon and a set of intracellular metal wire electrodes, for stimulation (S) and recording (R) are introduced through the distal cannula; when an accurate (D.C.) potential measurement is required, a calomel electrode is used. In this instance external stimulation is employed. B: A single glass capillary provided with a long slit on one side is inserted to the extent that the slit is approximately half way through the axon and the tip protrudes through the end; external stimulation (S) across a vaseline gap and internal recording by means of a microelectrode pushed through the membrane are employed. C: Two separate cannulae overlapping for about 20 mm in the middle portion of an axon, are used in conjunction with a microelectrode and external stimulating arrangements; the perfusate flows out of one cannula and is drained by the other. (Drawing not to scale.)

In C the perfusate was ejected through the opening of one of two overlapping (by about 20 mm) pipettes and collected through the opening of the second pipette. In another method, not illustrated in the figure, the perfusate flowed back toward the opening of the axon through which the perfusing glass capillary was introduced. In our technique there was always at the beginning a layer of axoplasm (50 μ or more) between the membrane and the zone of flowing fluid. Continuous perfusion usually resulted in erosion of the axoplasmic wall and in an appreciable reduction in its thickness.

Using isotonic KCl as a perfusate, the action potential remained normal for a period of approximately 30 min. This finding appeared to indicate that the internal sodium concentration could be reduced without changing the amplitude of the action potential. Conduction block was usually (but not always) preceded by prolongation of the duration of the action potential. Upon perfusing with sodium salt, instead of potassium, a rapid fall ensued in the resting potential and in the action potential amplitude. Using solutions of the same salt (Na or K) and fixing the concentration ratio across the membrane (with little adhering axoplasm) the membrane potential was found to depend on the level of the concentration ratios. Though the experiments were preliminary, this finding was interpreted as indicating the existence of fixed charge in the membrane (TEORELL, Progr. in Biophys. and Biophys. Chem., 3, 305, 1953). The axons employed were 400—600 μ in diameter.

Quite recently BAKER, HODGKIN and SHAW (cf. above reference) reported that they could remove nearly all of the axoplasm in their perfusion experiments. We cannot with certainty point to a particular difference in respective techniques that could account for their being more successful in maintaining the excitability for longer periods of time.

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From the Zoophysiological Institute, University of Lund, Sweden

Photometric Estimation of the Relative Amount of DNA in Early Chick Embryos

By

HADAR EMANUELSSON

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Abstract

EMANUELSSON, H. *Photometric estimation of the relative amount of DNA in early chick embryos.* Acta physiol. scand. 1961. 52. 197—210. — Using the two-wavelength method the author has determined photometrically the amount of DNA in interphase nuclei of cells from chick blastoderms, 0—40 hours of incubation. The investigation was performed on sectioned material, stained according to the Feulgen method. Comparison of results for the different developmental stages shows that essentially only minor variations exist between the stages. Exceptions are the yolk endoderm cells at the 40-hour stage which apparently are polyploid and cells from 5-hour blastoderms which have an average of 40 per cent more DNA, than that found in other stages. In combination with previous observation of a high level of DNA in early chick blastoderms the present result indicates a temporary occurrence of cytoplasmatic DNA in the chick embryo. The high DNA value at the 5-hour-stage is interpreted as indicating a partial synchronism in cell-division during the early development of the embryo.

In a previous investigation, EMANUELSSON (1958) the author has reported indications of a remarkably high amount of DNA in the cells of the early chick embryo. Thus for the first 18 hours of development it seems that the chick blastoderm has an average amount of DNA per cell in the embryo area which is double the amount recorded for cells from the same area later in development. As the analyses gave no information concerning the localization of this exceeding amount of DNA, it remained to be settled whether the high level of DNA reflects a widespread polyploidy in the early embryo cells or whether it possibly can be ascribed to a temporary occurrence of cytoplasmatic DNA.

As for the latter so many indications of the occurrence of cytoplasmatic DNA have been reported in the literature up to now that its existence scarcely can be

questioned any longer Little is known about the chemical nature of it the cytoplasmatic DNA reported for the amphibian egg is in fact thought to be desoxyribosidic compounds acting as DNA precursors, HOFF JØRGENSEN and ZEUTHEN (1952) GREGO and LØVTRUP (1955) BIEBER et al (1959)

Polyploidal cells, on the other hand, are not uncommon in animal embryos, as has been revealed by photometric investigations on isolated nuclei Special attention may be called here to MOORE's (1957) investigation on frog embryos in which she demonstrates a striking polyploidy in her material, there being represented nuclei with up to the octoploid amount of DNA.

In early chick embryos, incubated under normal conditions, the present author has *occasionally observed tripolar spindles in the dividing cells, but* obviously there is a very low frequency of these Otherwise there have been no cytological indications of polyploidy in these embryos.

To elucidate the question and settle whether an elevated DNA-level in the earliest stages can be attributed to an especially high amount of nuclear DNA or not it was finally decided to compare chick embryos at different stages of the early development with reference to the amount of nuclear DNA in the cells. For that reason photometric determinations of nuclear DNA have been performed on cells from early blastoderms, which after sectioning were stained according to the FEULGEN method

Material and Methods

Preparation of the material

The experimental material employed was *fertilized eggs of pure breed* (White Leghorn) laid the day before.

In some of the eggs the blastoderm was removed prior to incubation and immediately fixed (0-hour-stage) Other eggs were incubated at 37.5 ± 0.5 C for various lengths *of time before removal and fixation of the blastoderms (5- 14—16- 18- and 40-hour* stages) Fixative absolute alcohol + glacial acetic acid (3 1) held t + 4 C. The classification of the developmental stages is made in accordance with the system of Hamburger and Hamilton (1951) Control embryos, their incubation extended to 14 days, *showed a quite normal development.*

The blastoderms were left in the fixative for 20 hours, then after dehydration and passage through methyl benzoate and benzene they were embedded in paraffin. They were serially sectioned t 8 and 12 μ respectively The Feulgen staining was made *according to LEUCHTENBERGER (1958)* the time of hydrolysis being set at 12 minutes. All slides were stained in direct succession, and care was taken to carry out an absolute uniform treatment of the slides. The mounting medium was DePeX (*Gurr*) The cover glasses as well as the slides used were of uniform thickness.

Many investigators have successfully used the Feulgen reaction for quantitative determinations, cf. LEUCHTENBERGER (1958) SWIFT (1953) LESSLER (1953) KURNICK (1955) But obviously the intensity of the stain can vary with the fixative, with the time of hydrolysis and with different methods of preparation of the stain. However it may be assumed that in the present investigation the preparations are not subject to such errors, but are all mutually directly comparable, as all blastoderms have been prepared identically and in direct succession.

Photometric Feulgen determination

Evaluation of relative amount of DNA (chromophore) in the nuclei is based on the two-wavelength method. For theoretical discussions of the latter see, e. g., ORNSTEIN (1952) and PATAU (1952) The advantage of the two-wavelength method compared with the single-wavelength methods is that the former is much less sensitive to a heterogeneous distribution of the chromophore to be measured; further it does not require the object in sharp focus. The two wavelengths chosen in the present investigation, when dealing with Feulgen-stained material have been 519 mμ and 570 mμ respectively Thus the photometric measurements on the pure Feulgen stain and on stained nuclei showed that these two wavelengths fulfil the requirements of the method, i. e., extinction t one of them is twice the extinction at the other

The photometric measurements on the investigated embryo-material have been carried out with the aid of microspectrophotometer (the details of which are given below)

Estimation of the total amount of chromophore in single nucleus involves securing the transmission values t the two wavelengths stated for a circular field including the selected nucleus. The diameter of this field, which may be regulated by means of the ocular-diaphragm, was held constant in all the measurements.

From the transmission values the relative amount of chromophore is then calculated according to PATAU'S formulae.

Thanks to MENDELSOHN (1958) who has published a very useful set of tables based on PATAU' formulae, actual calculation is made unnecessary as the amount of chromophore can be derived in the tables directly from the transmission values. According to the same author only those values are included in the tables which fall within the effective range of the two-wavelength method. The transmission values found in the present investigation have all been within the scope of the tables in question.

The photometric measurements extended over many days. Therefore, as a precaution, samples of nuclei representing all the different stages were finally measured in rapid succession over short intervals. The latter values were in strict conformity with earlier results.

Micro-spectrophotometer

The micro-spectrophotometer used is of conventional design and based on the same principles as described by e. g LEUCHTENBERGER (1958) SWIFT and RASCH (1956) and MENDELSOHN (1958)

Light source. A Philips tungsten ribbon-filament lamp (10 V 16—17 Amp) operated at 15 Amp was the light source used. The light goes via flint-prism monochromator (Carl Zeiss, Berlin-Steglitz) the exit slit of which is replaced by a Pulfrich diaphragm (Zeiss, Jena) projecting square lightspot via a mirror and an achromatic sub-stage condensor in the image plane.

Optics The microscope was Zeiss-Winkel standard microscope equipped with an achromatic sub-stage condensor (COOKE, TROUGHTON and SIMMS n. a. 1.0) operated at low aperture (c. 0.3) For orientation in the slid the monochromatic light can be replaced by a built-in light-source. Objective used C. Zeiss immersion-objective (Zeiss Apo 100/1.32 Öl) Immersion oil refractive index 1.51.0. A Ramsden-ocular with built-in iris-diaphragm makes it possible to limit the area projected on the photo-cell.

Light-registration. Over the microscope a micro-photography camera bellows is adjusted in a horizontal swinging position and t registration the light is projected on photo-cell (RCA1P21) placed on top of the bellows. For centering the light spot on the photo-cell, the photo-cell-housing can be replaced by ground-glass plate. The photo-cell is fed by high-voltage stabilisator in our case operated at 960 V and readings are made on Multiflex spot-galvanometer

Main supply. All electric equipment is connected to the main supply via a Philips-stabilisator (500 VA) giving an output voltage of 220 ± 1 V at 195—255 volts input.

Actual tests with the photometric equipment have shown that the Schwarzschild-Villiger effect, NAORA (1952) is negligible in the present investigation.

It is difficult to estimate the errors associated with the two-wavelength method, as yet comparatively few investigators have applied this method for DNA-measurements and consequently sufficient material for a direct comparison is not available. Measurements undertaken by MENDELSOHN (1958) and MENDELSOHN and RICHARDS (1958) seem to indicate however that errors inherent in the two-wavelength method are indeed very small, and it may be assumed that they fall below the error margin of ordinary photometric DNA-measurements at a single wavelength. To the latter type of measurement a maximal error of 15 per cent has been ascribed, SWIFT (1953), LEUCHTENBERGER (1954).

At no stage was staining of the cytoplasm observed. The measurements have shown that not even at the earliest stages (0-hour and 5-hour blastoderms) when yolk granulae in the cells are very apparent, is there any measurable variation in amount of chromophore which may possibly be ascribed to the presence of these granulae.

In a sectioned material there is risk of including slightly injured or overlapped nuclei among those selected for measurement. However, provided the diameter of the cell nuclei is of approximately the same size for all the investigated stages — and on the whole that seems to be the case for the cells of the embryo area — this error will be of fairly constant size for sections of the same thickness. The mean DNA-values of blastoderms sectioned at 8 μ have constantly been c. 5 per cent lower than in blastoderms sectioned at 12 μ. The difference reflects the higher percentage of injured nuclei occurring in the thinner sections, cf. [illegible] (1960).

Results

All measurements are made on interphase nuclei. For each of the investigated embryo stages the individual DNA values (chromophore values) have been arranged in histograms which are represented in Fig. 1—3. The mean DNA value is given as well. The histograms refer to blastoderms sectioned at 12 μ. Each histogram is made up from values obtained from 2—3 blastoderms which among themselves showed closely agreeing distribution. As it is seen the individual nuclear values differ considerably but even if a minor part of the variation must be assigned to errors inherent in the applied methods, it may be confidently assumed that the found differences essentially reflect a real variation in DNA-content of the nuclei.

0-hour-stage

At this stage which forms a resting period before the onset of incubation, activity in the blastoderm is low and mitotic figures are sparse. Even if the found DNA values are considerably scattered, there is in the histogram (Fig. 1) an accumulation of values around the chromophore value 180 as well as another slightly smaller accumulation around the value 300. Of these the former may be considered as representing the mean DNA-value of the resting interphase nucleus, i. e., the mean diploid value.

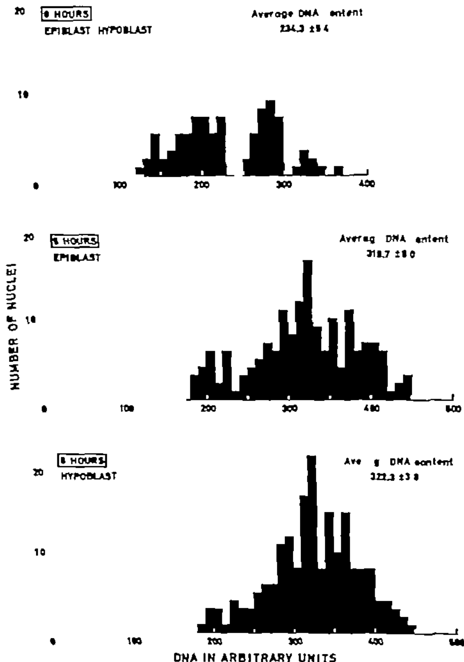


Fig. 1 Distribution of DNA-values in chick blastoderms sectioned at 12 μ.

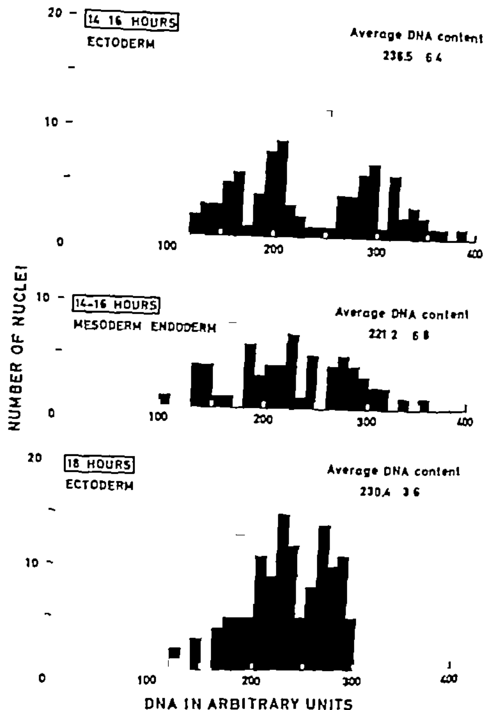


Fig. 2. Distribution of DNA-values in chick blastoderms sectioned at 12 μ.

As it has been shown by SWIFT (1953) and others doubling of the amount of nuclear DNA occurs in interphase. It seems reasonable to suppose that in the histograms values higher than 180 but below 360 are obtained from cells gradually preparing for mitosis. Now the physiological zero point for chick embryos is generally considered to lie at 20—21 C, EDWARDS (1902). However metabolic processes undoubtedly occur in chick blastoderms also at temperatures below this point, even if the actual process of cell-division seems to be strongly suppressed.

The sparse occurrence in the histograms of values two times the supposed diploid mean value fits well with the low frequency of mitotic figures. Cf. Table I

There exist no differences in the amount of nuclear DNA between epiblast (ectoderm cells) and underlying cells (hypoblast or endoderm) at this stage.

5-hour-stage

The picture given by the two histograms (Fig 1) is at first somewhat puzzling, as the mean DNA-value is of quite another magnitude than that met with in other developmental stages.

To account for this we must first consider that during the stage in question a general reorganization to a new metabolic equilibrium is going on in the embryo. Mitotic activity which until recently was suppressed by the low environmental temperature has started again to a full extent, and there is reason to believe that the majority of cells are preparing to enter cell-division.

The same conclusion will be arrived at from the histograms if we persist in considering the mean diploid value as ranging around 180. In the histograms major accumulations of values are thus found around the chromophore values 300 and 400 respectively and only a minor concentration is found around 200. The higher values may be taken as representing widespread DNA-duplication immediately preceding the actual cell-division.

The possibilities for the embryo to bring out such a general, almost synchronous DNA-duplication will be discussed later

As for the 0-hour-stage both germ layers show the same distribution of the DNA-values.

14—16-hour stage

18-hour stage

The histograms (Fig 2 and 3) are essentially of the same shape as for the 0-hour-stage, but a more pronounced concentration of values around the 180 value is found for the underlying layers.

Nuclei with the suggested tetraploid amount of DNA are almost lacking, but a significant number of the nuclei are just increasing their amount of DNA before mitosis.

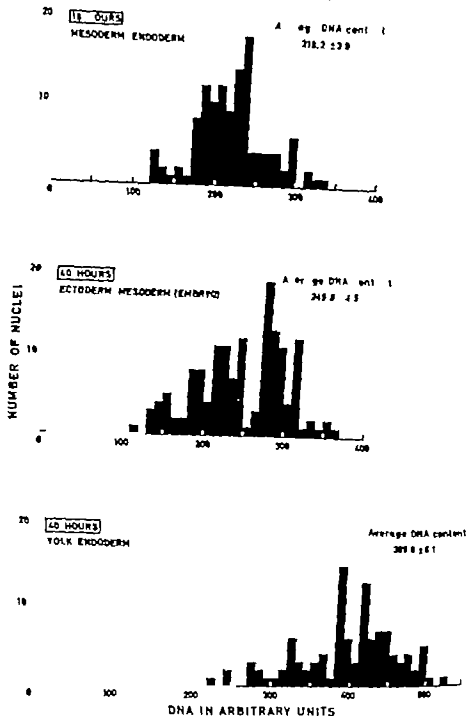


Fig. 3 Distribution of DNA-values in chick blastoderms sectioned at 12 μ.

Table I Mitotic activity in early chick embryos

Mitotic counts were made in the same sections, 12 μ thick, that first had been submitted to photometric estimation of nuclear DNA. To avoid double counting alternate sections were omitted in the determination. The standard error of the mean has been calculated according to the formula $\pm \sqrt{\frac{p(100-p)}{N}}$, where p = mitotic index, N = number of investigated cells. Reported indices are in all cases derived from 2 separate blastoderms, which among themselves showed closely agreeing values.

Age of the embryo	Tissue	Mitotic index (per cent)
0 Hours	Epiblast + Hypoblast	0.79 ± 0.14
5 Hours	Epiblast	3.62 ± 0.30
	Hypoblast	3.80 ± 0.36
18 Hours	Ectoderm	3.72 ± 0.26
	Mesoderm + Endoderm	2.27 ± 0.22
40 Hours	Neural tube	4.70 ± 0.40
	Yolk endoderm	1.49 ± 0.26

40-hour stage

At this advanced stage of development (Fig 3) the embryo area still shows much the same pattern as was found in 0-hour and 14—18-hour blastoderms, but cells from the extraembryonic portion of the blastoderm present an entirely diverse pattern with a mean DNA-value near 400 Further inspection of the extraembryonic cells proved that these higher DNA-values are restricted to yolk endoderm cells, whereas the pattern for the ecto- and mesoderm cells is essentially the same in the extraembryonic as in the embryo area.

Except for this last mentioned stage (40 hours) distribution of DNA values within a germ layer is apparently much similar for the embryonic and extraembryonic portion of it.

The main result obtained is that during the investigated period of the development the chick embryo cells hold practically the same average DNA content in the nuclei. The exceptionally high DNA-level at the 5-hour-stage is possibly of short duration.

To find out how close a connection there may be between distribution of nuclear DNA and apparent mitotic activity in the analyzed sections, mitotic indices have been calculated for some of the blastoderms. These values are given in Table I It appears that mitotic indices are not differing when the germ layers show the same distribution of DNA-values (5-hour-stage) If the distribution is apparently dissimilar however the indices deviate too (18-hour

stage) The higher index is then found in the layer having the higher portion of nuclei in the midst of DNA-synthesis. In the 18-hour blastoderm the differences between the germ layers in mitotic index and DNA-distribution possibly suggest a prolongation of interphase in meso- and endoderm cells as compared with the ectoderm cells.

Obviously the comparatively low mitotic indices (3.6—3.8 per cent) for the 5-hour-stage are difficult to account for in the present case, as they were expected to be definitely higher than any value for the 18-hour-stage. Preliminary investigations indicate however that slightly older embryos (estimated at 5—8 hours) have a somewhat higher average value for the epiblast (about 5 per cent) Presumably this small but apparently significant increase bears direct relation to the deviating DNA-distribution met with in the 5-hour blastoderm.

Discussion

The theory of DNA-constancy according to which the amount of DNA per chromosome set is constant for an organism, has won widespread acceptance and Swift (1953) in his review on the subject concludes that the theory has been adequately confirmed He admits, however that the constancy in question may break down during the differentiation of cells. Discussing her own results from DNA-measurements on nuclei from frog embryo hybrids, Moore (1957) stresses the fact that the DNA-constancy has not been established for embryonic cells and quotes results from vertebrate as well as invertebrate embryos.

In the present investigation the histograms are of an essentially similar shape, consistent with the constancy theory However the low frequency of high (tetraploid) DNA values — especially for the 14—18-hour-stage — is confusing Otherwise the shape of the histograms — with a main peak at the diploid value and a lower subsidiary peak at twice this value — is apparently what is to be expected in rapidly proliferating tissues, Bloch (1958)

Two stages, the 5-hour-stage and the 40-hour stage show some peculiar features, which require further comment.

At the 40-hour-stage the yolk endoderm cells with their mostly tetraploid amount of DNA in the nucleus stand out distinctly against the cells of the embryo body Along with the tetraploid value higher and also lower values are represented, but the shifting towards higher values is predominant and, strikingly enough nuclei with the diploid amount of DNA are missing

Closer inspection of the yolk endoderm cells in mitosis revealed that all the mitotic figures observed differ in some way from the normal type, often displaying apparently defect chromosomes. Mitotic disturbances in the yolk endoderm cells have earlier been reported by Jacobson (1938) and also in his opinion the abnormal mitoses are a characteristic feature of the cell type in question. Judging from the investigated slides, the whole layer of yolk endoderm cells will most likely degenerate ultimately a phenomenon which may

bear some relation to the simultaneous outgrowth of the vascular system. As to the nature of the mechanism that prevents the endoderm cells from cell-division without blocking their DNA-synthesis, nothing definite seems to be known. However cell degeneration, often on a large scale, is apparently a normal phenomenon in vertebrate embryos, GLÜCKSMANN (1951) Substances released in cell degeneration in embryos probably exert an inducing effect, STOCKENBERG (1937)

Interestingly enough a cytological picture, very similar to that in the yolk endoderm at the 40-hour-stage, was observed by the author in the extraembryonal portion of 18-hour-blastoderms upon treatment with ribonuclease (RNA-se solution injected in the egg directly under the blastoderm)

The remarkable distribution of DNA-values at the 5-hour-stage with most of values located above the diploid level has been interpreted here as indicating that DNA-duplication is nearly synchronous in the majority of the blastoderm cells. The possibility of there existing instead just at the 5-hour-stage a population of nuclei *with 300 as the mean diploid value seems less plausible.* The distributional patterns of DNA-values from preceding and succeeding developmental stages (0-hour and 14—16-hour-stage) lend no support for such an assumption.

However a synchronous DNA-duplication on a large scale, as just outlined, can scarcely be accomplished by the blastoderm cells at this early stage without access to either stored DNA or stored desoxy-compounds, directly utilizable in the DNA-synthesis. The latter would rather be of desoxyribonucleoside-type, as according to KORNBERG (1959) the immediate precursors for DNA-synthesis are desoxyribonucleoside triphosphates.

In the investigation that was referred to in the introduction, EMANUELSSON (1958) the author has shown that calculation of the average amount of DNA per cell for the embryo area in early chick blastoderms gives values for the 0-hour-stage and the 18-hour-stage which far exceed the value for the 26-hour stage. The latter value, as well as values for more advanced stages are, however in complete accordance with average values for chick embryos, reported by other investigators, e. g DAVIDSON and LESLIE (1950)

The higher values alluded to above were obtained from biochemical analyses in combination with meticulous counting of cells of the corresponding area, but they were unfortunately rather incomplete. However additional observations have given similar results, Fig 4 and therefore the found deviation has been considered reliable.

As the present investigation has shown that the average amount of DNA per nucleus is practically the same for the 0- 18- and 40-hour-stages, it is concluded that an elevated amount of DNA at the earliest stages probably is due to a stored, extra-nuclear supply of DNA. Even if this supply is rather small at the 0-hour-stage, it seems just big enough to permit such an almost simultaneous cell-duplication at about the 5-hour-stage as is now suggested. A temporarily

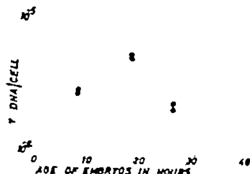


Fig. 4. Average amount of DNA per cell in the embryo area of early chick blastoderms The values are derived from biochemical analyses and cell counts for the area in question For further details cf. EMANUELSSON (1958).

raised demand for DNA, which was to be foreseen then, is also indicated in figure 6 by the decrease of the DNA level at about the actual stage.

As is seen in Fig 4 there follows upon the decrease a replenishment of DNA to the original level presumably effected by stored DNA-precursors but from the 26-hour-stage on the exceeding amount of DNA is apparently exhausted and the embryo has switched over entirely to DNA-synthesis de novo It seems likely that the last DNA reserve is being used during that period of increased mitotic activity which in fact occurs in the embryo area at about the 20-hour stage.

From HOFF JORGENSEN's (1954) investigations it is known that the whole hen's egg is rich in DNA Furthermore the proposed scheme of an altered DNA metabolism in the early chick embryo has its direct parallels. It is known that a shifting during embryogenesis from consumption of stored DNA or immediate DNA-precursors to DNA-synthesis de novo occurs in frog embryos, HOFF JORGENSEN and ZEUTHEN (1952) GRANT (1960) and in sea urchin embryos, AGRELL and PERSSON (1956) In the latter case it was indeed demonstrated that the embryo cells divided synchronously up to that developmental stage when the DNA-supply was exhausted and synthesis de novo started.

The figures in table I show that the mitotic index gives little support to the proposed scheme of a partial accumulation of cell division to the 5-hour-stage. However it is difficult to predict how much the absolute value of the mitotic index may be influenced from the shifting DNA-distribution as we do not know the length of the period during which interphase nuclei have a DNA-content, twice the diploid amount. It seems likely that the mitotic index is not much affected if the last mentioned period is appreciably longer than the time for mitosis — a condition which possibly prevails at the 5-hour-stage.

Circumstantial evidence in favour of a partial synchronism in cell division in early chick embryo cells has also been reported earlier EMANUELSSON (1958) and include principally a) rhythmical variation in the average DNA- and RNA-concentrations of early chick embryo cells, b) a varying DNA-synthesizing ability of early embryos, explanted at different stages.

The possible existence of a periodicity of cell division in chick embryos has been discussed by earlier investigators, of whom STOUGH (1935) may be especially mentioned. In their opinion there is no evidence of any periodicity. However this view of the question is easily explained by the fact that the investigated embryos almost invariably have been older than 20 hours. Thus a highly important period actually has been missed.

DERRICK (1937) in her investigation of the mitotic index in the early chick embryos has drawn no conclusions regarding periodical variations in the average mitotic index, but valuable information bearing just upon this problem can be obtained from her values. They reveal that for all germ layers the average mitotic index is noticeably high in embryos at about the 4-hour, the 20-hour and the 5—8-somite-stage respectively. This finding is apparently in fairly good agreement with a suggested periodicity of cell division in the chick embryo.

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References

AGRELL, I. and H. PERSSON, Changes in the amount of nucleic acids and free nucleotides during early embryonic development of sea urchins. *Nature* (Lond.) 1956. *178*. 1398—1399.

BIEBER, S., J. A. SPENCE and G. H. HITCHINGS, Nucleic acids and their derivatives and the development of Rana pipiens. *Exp. Cell Res.* 1959. *16*. 202—214.

BLOCH, D. P. In "Frontiers in cytology". Ed. by S. L. PALAY. *New Haven* 1958. 113—166.

DAVIDSON, J. N. and I. LESLIE, A new approach in the biochemistry of growth and development. *Nature* (Lond.) 1950. *165*. 49—53.

DERRICK, G. E., An analysis of the early development of the chick by means of the mitotic index. *J. Morph.* 1937. *61*. 257—284.

EDWARDS, C. L., The physiological zero and the index of development of the eggs of the domestic fowl, Gallus domesticus. *Amer. J. Physiol.* 1902. 6. 351—397.

EMANUELSSON, H., Variations in nucleic acid concentration during the development of early chick embryos. *Acta physiol. scand.* 1958. *44*. 336—364.

GLÜCKSMANN, A., Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* 1951. 26. 59—86.

GRANT, P. The influence of folic acid analogs on development and nucleic acid metabolism in Rana pipiens embryos. *Dev. Biol.* 1960. 2. 197—251.

GREGG, J. R. and S. LØVTRUP, Synthesis of desoxyribonucleic acid in lethal amphibian hybrids. *Biol. Bull.* 1955. *108*. 29—34.

HAMBURGER, V. and H. L. HAMILTON, A series of normal stages in the development of the chick embryo. *J. Morph.* 1951. *88*. 49—73.

HOFF-JØRGENSEN, E. in KITCHING: "Recent developments in cell physiology". London 1954.

HOFF-JØRGENSEN, E. and E. ZEUTHEN, Evidence of cytoplasmic deoxyribosides in the frog's egg. *Nature* (Lond.) 1952. *169*. 245—246.

IVERSEN, S., Visual observations on the localization of DNA and nucleoli in somatic cell nuclei. *Acta anat.* (Basel) 1960. *41*. 160—172.

JACOBSON, W. The early development of the avian embryo I—II. *J. Morph.* 1938. *62*. 415—501.

KORNBERG, A., Enzymatic synthesis of deoxyribonucleic acid. *Harvey Lect.* 1959. *53*. 83—112.

KURNICK, N. B., in "Int. Review of Cytology" Ed. by BOURNE, G. H. and J. F. DANIELLI, New York 1955. IV. 221—268.

LESSLER, M. A., in "Int. Review of Cytology" Ed. by BOURNE, G. H. and J. F. DANIELLI, New York 1953. II. 241—247.

LEUCHTENBERGER, C., Critical evaluation of Feulgen microspectrophotometry for estimating amount of DNA in cell nuclei. *Science* 1954. *120*. 1022—1023.

LEUCHTENBERGER, C., in "General Cytochemical Methods" Ed. by J. F. DANIELLI, New York 1958. I. 219—278.

MENDELSOHN, M. L., The two-wavelength method of microspectrophotometry I—II. *J. Biophys. Biochem. Cyt.* 1958. *4*. 407—431.

MENDELSOHN, M. L. and B. M. RICHARDS, A comparison of scanning and two-wavelength microspectrophotometry. *J. Biophys. Biochem. Cyt.* 1958. *4*. 707—709.

MOORE, B. C., DNA in diploid and androgenetic amphibian embryos. *J. Morph.* 1957. *101*. 227—274.

NAORA, H., Schwarzschild-Villiger effect in microspectrophotometry. *Science* 1952. *115*. 248—249.

ORNSTEIN, L., Distributional error in microspectrophotometry. *Lab. Invest.* 1952. *1*. 250—265.

PATAU, K., Absorption microphotometry of irregular-shaped objects. *Chromosoma* (Wien) 1952. *5*. 341—362.

STOCKENBERG, W., Die Orte besonderer Vitalfärbbarkeit des Hühnerembryos und ihre Bedeutung für die Formbildung. *Arch. EntwMech. Org.* 1937. *135*. 408—425.

STOUGH, H. B., Further studies in modified mitosis. *J. Morph.* 1933. *58*. 221—256.

SWIFT, H., in "Int. Review of Cytology" Ed. by BOURNE, G. H. and J. F. DANIELLI, New York 1953. II. 1—76.

SWIFT, H. and E. RASCH, in "Physical techniques in biological research" Ed. by OSTER, G. and A. W. POLLISTER, New York 1956. 353—400.

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From the Zoophysiological Institute, University of Lund, Sweden

Mitotic Activity in Chick Embryos at the Primitive Streak Stage

By

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Abstract

EMANUELSSON, H. *Mitotic activity in chick embryos at the primitive streak stage.* Acta physiol. scand. 1961. 52. 211—233. — In the present work it has been investigated whether previously recorded regional differences in the average RNA-concentration of the primitive streak embryo are also connected with differences in mitotic activity. Mitotic indices calculated for the embryo area reveal no decided regional dissimilarities. However, owing to the uncertainty inherent in the interpretation of the index, it was decided to extend the mitotic counts to embryos in which successive accumulation of mitoses had been produced by means of heat shock and the mitotic poisons, colchicine and oestradiol. From the obvious differences in mitotic activity which appear in such embryos it is clear that cell multiplication is greatest in a region of the embryo comprising the node and adjacent areas on both sides thereof. As differences in cell multiplication are moderate, however, it is presumed that the existing variation in RNA-concentration must be partly ascribed to differences in protein synthesis within the embryo, associated with the beginning differentiation of the embryo cells. Cytologic and morphologic changes caused by the heat shocks and the mitotic poisons are described.

Much work has been devoted to the study of morphogenesis in the early chick blastoderm, and the numerous papers dealing with this subject have recently been excellently reviewed by WADDINGTON (1952). Most investigators have studied the large-scale cell-movements, usually referred to as "streaming" which come into operation in the early blastoderm, but the mitotic activity occurring at the same time, has also attracted considerable interest. However

to what extent localized cell proliferation can be involved in the outgrowth of the embryo body does not seem very clear. Remarkably enough, the rapidly growing primitive streak, for instance, cannot possibly be considered as a center of cell proliferation (PASTEELS 1913).

Describing variations in the concentration of nucleic acids in the early chick embryo, the present author (EMANUELSSON 1958) has demonstrated within the embryo area the existence of local variations in the quotient RNA/DNA (an expression for the relative concentration of RNA), which according to the author suggests *variations in cell multiplication.* Thus low values of the quotient would denote lively cell-division while high values of the quotient would signify a lower mitotic activity, possibly with predomination of cell-differentiation within the region in question.

For chick embryos of 23—40 hours incubation this relation between the RNA/DNA quotient and the mitotic activity apparently exists. This relation is substantiated by preliminary observations of the present author, from metabolic data (SPRATT 1952) and from mitotic indices, reported by e. g. DERRICK (1937) and BELLAIRS (1955, 1957).

Of great interest is the question of whether the relation holds also for the primitive streak embryo (18-hour-embryo), in which a visible differentiation has not yet occurred, even if the presumptive fate of the different regions already is well defined, cf. WADDINGTON (1952).

In fact, the mitotic indices reported by DERRICK (1937) for chick embryos of this age lend some support to the idea of a variation in mitotic activity such as intimated from the nucleic acid quotients. However, DERRICK's indices for the primitive streak stage — up to now the only existing detailed information of the mitotic activity in the 18-hour chick embryo — can scarcely be adopted in the present case without some reservation. Even if they often indicate big differences between adjacent regions, it is difficult to judge when a difference is significant, as statistical analysis of the material is lacking. Moreover, these indices are calculated for each germ layer separately, whereas the RNA/DNA quotients referred to were obtained for pieces of blastoderm containing all the three germ layers.

It has therefore been of interest to make a further investigation of mitotic activities in the primitive streak embryo in order to obtain *mean values* for cell multiplication within small, restricted sections of the blastoderm. In the present investigation the position of these sections has been of primary interest, while their composition of cell material from the different germ-layers has been of secondary importance.

As it is fully recognized that a local increase of the mitotic activity is not bound to result in an appreciably elevated mitotic index — even if that seems likely — attempts have also been made to obtain additional measures of the mitotic activity in the blastoderms, more elucidative than the ordinary mitotic index.

The present paper deals principally with experiments in which blockage of blastoderm cells, having entered mitosis, will lead to successive accumulation of mitotic figures and thus facilitate the discovery of any existing differences in cell multiplication. In the experiments the mitosis process has been blocked by heat and by addition of mitotic poisons, colchicine and oestradiol.

Material and Methods

Incubation of the eggs (White Leghorn, pure breed) was carried out in a thermostatically controlled incubator at 37.5 C ± 0.5 C. The atmosphere of the incubator was kept suitably moist by placing dishes of water inside.

Normal Embryos

After incubation for the desired period the embryos were removed from the eggs and were immediately fixed in Carnoy' fluid (bsolute alcohol + glacial acetic acid, 3 1) They were stained in Gomori's hematoxylin according to MELANDER and WINGSTRAND (1953) and were mounted in DePeX (Gurr) Some of the embryos were sectioned (10 μ) but usually they were mounted whole.

Heat-Shocked Embryos

Preliminary experiments had proved that the mass of hen's egg is too big to permit rapid changes in temperature of the egg contents, a fact which precludes the possibility of short-time incubation of the eggs at widely differing temperatures. All experiments including variations of the incubation temperature of the embryos have therefore been performed exclusively upon embryos cultivated in vitro in Carrel-flasks of small volume. To ensure even temperatures incubation was arranged in a thermostatically controlled water-bath, the flasks being almost entirely submerged in the water Changes of the water temperature could be made rapidly· thus alternation between two widely differing temperatures, e. g 20° C and 44 C, was accomplished in about 3 min. The embryos were explanted to the Carrel-flasks at the primitive streak stage (18 hours of incubation according to the system of HAMBURGER and HAMILTON (1951)). The explants have constituted the anterior part of — alternatively the whole of — the embryo area, the latter being defined in an earlier investigation, EMANUELSSON (1958) (cf. also Fig. 1). They were cultivated on basic medium of the following composition

	per cent
NaCl	0.650
KCl	0.033
$CaCl_2$ 6 H_2O	0.031
Na_2HPO 2 H_2O	0.007
KH_2PO	0.003
$NaHCO_3$	0.035
Glucose	0.850
Agar	0.420

Before addition of the agar egg white was added 8 parts of basic medium were mixed with 2 parts of solution prepared by shaking one egg white vigorously with 50 ml medium. By addition of egg white to the medium it was possible to maintain satisfactory mitotic activity for up to 48 hours in the explants. The explants were fixed in Carnoy's fluid or methanol, as stains were used Gomori' hematoxylin, gallocyanin-chromalum and Giemsa solution. Mounting medium: DePeX.

Treatment with Oestradiol and Colchicine

In the treatment *in vitro* explanted embryos were cultivated on the basic medium, described above (no egg white) to which was added oestradiol or colchicine. As before the embryos were explanted at the primitive streak stage the explant constituting the whole embryo area. Incubation was carried out at 37.5 C ± 0.5 C.

All experiments in vitro — also the heat-shock experiments — always have included controls, cultivated on the employed medium at 37.5 C.

For oestradiol a suitable concentration proved to be 11 μg/ml incubation medium. When adding the oestradiol to the basic medium it was necessary first to dissolve it in ethanol. Controls showed that the resulting very low concentration of ethanol of the medium (0.19 per cent) had no effect upon the cultivated embryos. With colchicine, which was dissolved directly in the medium, full effect was obtained in a concentration of 100 μg/ml cultivation medium.

Treatment with mitotic poisons *in ovo* was arranged so that after rubbing the egg with a pellet of cotton soaked in 70 per cent ethanol, a small opening was made in egg shell and shell membranes on the side of the egg, whereupon the drug, dissolved in 0.3 ml sterile 0.93 per cent NaCl-solution (37° C) was injected into the yolk, directly under the blastoderm. By using a curved hypodermic needle of small calibre it was possible to avoid piercing the vitelline membrane in the immediate vicinity of the blastoderm, and there was almost no escape of yolk out into the egg white. After the hole in the shell had been sealed with tape the egg was immediately restored to the incubator and placed with the "window" pointing sideways. Controls were injected with sterile NaCl-solution only.

Treatment in ovo was successful as far as it concerned colchicine, which was added in a dilution of 1 : 10^4.

When using oestradiol in this type of experiment it was found that the concentration thereof had to be raised considerably over that used in the experiments in vitro. As it appeared that the concomitant increase in the ethanol concentration obviously had undesirable effects upon the embryo cells further experiments were suspended.

After treatment with mitotic poisons the embryos were fixed in Carnoy's solution, stained in Gomori's hematoxylin and mounted in DePeX. The embryo material comprised both sections (10—12 μ) and whole mounts.

Results

A. Mitotic Indices in Normal Chick Blastoderms at the Primitive Streak Stage

In Table I are given mitotic indices for normal chick embryos of 16—23 hours incubation. As seen the figures refer to the whole embryo area as well as restricted parts thereof. For the primitive streak embryo (18-hour-embryo) the extent of these parts is outlined in Fig. 1.

The table permits comparison both between transversal and longitudinal strips of the embryo area. The transversal strips are numbered from the fore part of the embryo so that part 1 includes the region before the streak, part 2 the anterior part of the streak with the node, and the remaining parts finally (3 and 4) comprise the central and terminal parts respectively of the streak. The 23-hour-embryo forms an exception to this: here the node is included in part 3. Three longitudinal strips are distinguished: one central and two lateral parts.

The mitotic index (the number of mitoses per 100 cells) reported for one of

Table I. Mitotic indices (per cent) for different parts of early chick embryos

The standard error of the mean is calculated according to the formula $\pm\sqrt{\frac{p(100-p)}{N}}$,

where p = mitotic index, N = number of investigated cells

Part of embryo. Cf. fig. 1 A	Age of embryos 16 hrs	16—18 hrs	18 hrs	18 hrs	20 hrs	23 hrs
1	2.83 ± 0.50	3.75 ± 0.27	5.0 ± 0.45	5.20 ± 0.26	4.50 ± 0.29	3.06 ± 0.20
2	3.22 ± 0.25	4.06 ± 0.20	5.17 ± 0.28	6.40 ± 0.29	4.65 ± 0.31	4.50 ± 0.22
3	2.39 ± 0.27	3.86 ± 0.26	5.15 ± 0.29	5.17 ± 0.25	6.15 ± 0.24	4.47 ± 0.18
4	2.02 ± 0.33	3.75 ± 0.28	4.47 ± 0.26	4.60 ± 0.25	5.31 ± 0.24	4.39 ± 0.21
$L_1 + L_2$	2.70 ± 0.22	3.62 ± 0.17	4.70 ± 0.21	5.02 ± 0.23	5.03 ± 0.20	3.75 ± 0.15
O	3.25 ± 0.27	4.13 ± 0.17	4.72 ± 0.20	5.75 ± 0.20	5.57 ± 0.17	4.20 ± 0.14
1 + 2 + 3 + 4	2.89 ± 0.17	3.89 ± 0.12	4.70 ± 0.14	5.47 ± 0.13	5.33 ± 0.13	4.15 ± 0.10

these parts represents a mean value for the whole body of cells within it, irrespective of connexion to any specific germ layer. Each of the mean values is based upon examination of a great many cells, usually 3,000—6,000. By suitable staining (Gomori hematoxylin) cells and mitotic figures located at shifting levels within the preparation could be counted directly without sectioning of the material.

For the primitive streak embryo biochemical analyses had shown that those parts of the embryo area which here are denoted 1—4 have the following RNA/DNA-quotients 7.3 4.9 7.1 8.5. If the previously mentioned relation between this quotient and mitotic activity (see introductory part) is prevailing at this developmental stage too, the above values would imply that mitotic activity is high in that region of the embryo surrounding the anterior part of the streak, but lower in posterior regions as well as in the region in front of the streak. Similar variations in mitotic activity are also expected in immediately preceding and succeeding embryo stages.

At first sight the found mitotic indices indicate such differences in cell multiplication within the 18-hour-embryo that would be well consistent with the variations of the quotients in question but statistical analysis reveals that the figures do not differ significantly (Table I). However there exists a real difference between part 2 and part 4 in the second of the two investigated 18-hour-embryos.

As for the mitotic spectrum, [illegible] the relative distribution of the various phases of mitosis among the dividing cells, it has not been possible either to demonstrate any profound dissimilarities between the parts.

Within the slightly younger embryos (16- and 16—18-hours) reliable differences between mitotic indices are lacking as well, but in the most advanced stages (the 20- and the 23-hour-embryo) the mitotic index of part 1 differs

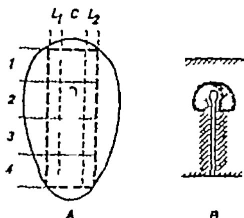


Fig. 1 A. Drawing showing the extension of the regions of the 18-hour-embryo, dealt with in table I. Transversal stripes: regions 1—4. Longitudinal stripes: regions L_1, C and L_2. Together the regions 1—4 comprise that part of the blastoderm which in the text is designated as embryo area. Embryo explants either constitute the whole of the embryo area or the anterior half thereof (region 1 + anterior half of region 2).
B. Schematic illustration of mitose-accumulation (= striation) in explanted 18-hour-embryos during the first mitotic wave after a series of heat shocks. High degree of accumulation is indicated by dense striation.

noticeably from the other values. This is especially pronounced in the last-mentioned embryo stage, for which the RNA/DNA-quotient of part 1 is actually 50 per cent higher than in parts 2—4.

The general impression from Table I of the primitive streak embryo is that cell multiplication within the embryo area is not as variable as was to be expected from the biochemical data. Notwithstanding there are strong tendencies towards regional differences just in the suggested direction, and they can scarcely be ignored.

Another important piece of information recorded in Table I is that among the investigated stages the average mitotic index for the whole embryo area will reach its highest values in the 18—20-hour-old chick embryo. Earlier findings (EMANUELSSON 1958) have proved that the RNA/DNA-quotient — calculated for the whole embryo area — attains a minimum just in the 20-hour-embryo. Thus for the whole embryo area, at least the inverse relation between RNA-content and mitotic activity is quite obvious.

In the account given above the estimation of the mitotic activity in the chick embryo has been based entirely upon records of the mitotic index.

Now the generally accepted method of proving the existence of a local cell proliferation in animal tissues is to demonstrate the local occurrence of a significantly higher mitotic index. However the mitotic index is considered to give the relation between the duration of mitosis and that of interphase, and it is obvious that no direct information is obtained about the generation time of the cells, i. e., time for mitosis + time for interphase (RIS 1955). Consequently it may be difficult to interpret the meaning of an elevated mitotic index correctly. If supplementary information about the investigated tissue is not at hand, e. g., about its normal histologic appearance and the normal duration of morphologic changes etc., it is, paradoxally enough, almost impossible to decide whether an elevated mitotic index is to be regarded as a manifestation of a stimulated or blocked cell multiplication. For further discussions about

the mitotic index, see e.g. WOODARD (1948) HOFFMAN (1949) and WALKER (1954)

Further it is easily understood that small differences in mitotic index are definitely proved only after the examination of a vast number of cells.

In the present situation the recorded variations of the mean values of the mitotic index, therefore, seemed worthy of further investigation. It was decided to try to arrest the embryo cells in mitosis and in that way bring about a successive accumulation of mitoses. Then if existent, even minor regional differences in mitotic activity ought to be revealed when comparing the relative accumulation in the various parts of the embryos.

B. *Treatment with Supra-Optimal Temperature*

In the first instance it was hoped to produce at least a limited accumulation of mitoses simply with the aid of a temporarily elevated incubation temperature.

In the ciliate Tetrahymena elevation of the environmental temperature may be so adjusted that the cell (animal) is just completely prevented from entering mitosis, other cell functions, however e. g., synthesis of nucleic acids, proceed uninterruptedly (ZEUTHEN and SCHERBAUM 1954) After reversion to the optimal temperature, cell division is started again in the previously blocked cells. As the number of blocked cells gradually increased at the elevated temperature, the result will be that when conditions are favourable for mitosis, a lot of cells will enter this state simultaneously Thus there will ppear a veritable wave of mitoses in the cell culture.

Now conditions are, of course, quite different for chick embryo cells than for Tetrahymena, thus, for instance the former have a much higher optimum temperature. Just owing to this fact it was at first doubtful whether mitosis could be blocked in chick embryo cells by heat treatment and then released en bloc without serious damage to the cells.

KEMP and JUEL (1931) have investigated the heat sensitivity of tissue cultures of the chick heart and report that incubation for 5 min at 47—50° C produced a prolonged, but reversible block of cell multiplication. At 45—46° C the block was less pronounced, whereas 5 min at 42—44 C, finally was almost without effect upon the mitotic activity In one single case a just discernible compensatory rise in the mitotic index was observed after the heat treatment.

DEUCHAR (1952) found remarkably good tolerance of primitive streak embryos in ovo towards such an extreme temperature as 45.5 C. Of chick embryos incubated at this temperature for 5 hours in ovo about 50 per cent appeared normal when examined 3—5 days later However as no allowance was made for warming of the eggs from room temperature to 45.5 C, the time for the actual heat shock must have been considerably less.

Single Shock

In the beginning of the present experiments, all of which have been made in vitro, embryos at the primitive streak stage were incubated at 45 0° C for

3.5 hours, whereupon incubation was continued for 0—24 hours at 37.5 C. Assuming the generation time of the cells to be at least 8 hours, cf. Rn (1955) the selected time for the treatment was considered adequate for effecting a distinct compensatory wave of mitoses, as the preliminary experiments had revealed that such an effect could be produced.

The embryo cells could just endure this treatment, but if the time was prolonged or if the temperature was raised further there appeared an increased number of pycnotic nuclei as well as pronounced necrosis afterwards.

Embryos examined *immediately after the treatment at 45.0° C* showed slight morphologic changes. However it was apparent in several cases that the primitive streak had broadened considerably Normal mitotic figures were entirely absent and during the treatment they were observed only immediately after the start of the experiment, when it was obvious that the cells were able to complete such mitoses that already were in progress.

The greater part of the nuclei were after their treatment in a state reminiscent of early prophase. In many cells there had occurred a conversion of the contents of the nucleus into many small heavily stained granula, a phenomenon, commonly observed in embryo cells undergoing mitosis at normal temperature after a previous heat treatment. This arrangement of the nuclear material is considered to correspond to advanced prophase. However it is assumed that the mitosis process cannot proceed any further at the high temperature as the nuclear membrane of the cells did not disappear

Here and there in the preparations a few pycnotic nuclei were to be found, and in fact, there appeared what may possibly be considered as very much deformed ana- and telophases, but they were very rare.

Staining with Giemsa's solution and with gallocyanin-chromalum revealed that the heat treatment led to a general disappearance of the nucleoli. Together with the observation of a fainter staining of cytoplasm in the treated cells, as compared with controls stained under exactly similar conditions, it indicates that the shock has seriously interfered with RNA-synthesis in the embryos

During *the following incubation at 37.5° C* embryos were collected at regular intervals and were subjected to morphologic and cytologic examinations.

Even when the inspected embryos had been incubated at normal temperature (37.5) no less than 24 hours after the heat treatment, normal morphology was not displayed in any case. Generally the primitive streak, when discernible, was much broadened During the period investigated no embryo exhibited a closed neural groove or distinct brain vesicles. Somites never appeared Obvious separation of the germ layers along the edges of the explant was noted.

All observations by the author indicate that the normally occurring streaming of the cells in the blastoderm at the primitive streak stage was severely disturbed.

The restoration of normal temperatures soon resulted in the recommencement of mitotic activity A great many cells that had been prevented from enter

ing mitoses owing to the inhibiting effect of the high temperature now completed this process simultaneously. As a result during the 24 hours at 37.5° C after the shock the previously comparatively even mitotic activity in the developing embryo was changed into a decided periodicity reflected in some big waves of mitoses. In fact minor waves between them were also recorded in some cases.

The first big wave comes shortly after the treatment has ceased, and it reaches its maximum about 2 hours after the transfer to the lower temperature. It is to be noted however that the different stages of mitosis that are exhibited now during the wave are not of the ordinary appearance.

In prophase distinct chromosome filaments are not to be seen, but instead the nuclei are filled with small granula, more or less distinctly arranged as strings of beads. Later when the nuclear membranes have disappeared and all the dividing cells have entered metaphase, the whole preparation takes on a very strange appearance, crowded as it is with round metaphase-chromosomes, many of which are widely scattered within the cells. Also the anaphase and telophase stages are confusing owing to the abnormally contracted form of the chromosomes. Under the existing circumstances calculation of the mitotic index was almost impossible but during the first wave it was estimated to amount to more than 30 per cent in the region around the anterior part of the primitive streak.

It was evident that the released mitoses were not evenly distributed in the explanted embryos. As far as it could be controlled, the mitotic index was especially high in the vicinity of the primitive streak, and mitoses were obviously more frequent in the ectoderm than in the underlying layers (meso- and entoderm).

The next big wave occurred about 8 hours later, i. e., when the embryos had been incubated for about 10 hours at 37.5° C. As well as during the first wave the prophase and metaphase stages show an abnormal appearance with the chromosome material still in the form of round granula, but at the end of this wave many normal ana- and telophases are distinguished. In many of the anaphases it is obvious that the bivalents have difficulty in accomplishing a separation, hence extremely extended anaphase chromosomes will result.

The third big wave during the investigated period came about 20 hours after the start of incubation at 37.5° C. Even if the embryonic material investigated is still characterized by cells with their chromosome material in the state of granula, the number of normal mitoses is conspicuously large. However surprisingly many tripolar spindles now appear among the anaphases.

Series of Shocks

In all further experiments in which chick embryos were exposed to high temperature shocks, the author tried to modify the treatment in order to reduce chromosome disturbances in the cells to a minimum without abolishing the

wave promoting effect. To attain such a result it proved necessary to lower the temperature of the shock and to shorten the duration thereof.

The appropriate temperature appeared to be 44.0° C. At this temperature most embryo cells were still prevented from entering mitosis, but if the temperature was lowered further (e. g., to 43° C) the resulting block was apparently very incomplete. As to the duration it should preferably be about one hour (at 44° C) if big waves of mitoses are to be effected. But even shocks of this length will cause serious disturbances to the embryos, preventing normal morphogenesis.

Eventually a more lenient treatment was arranged so that embryos were exposed to a *series* of heat shocks (44.0° C) alternating with periods when the incubation temperature was lowered to 20° C. A duration of 30 minutes was chosen both for the shocks and for the intervals between them. *By keeping the* temperature as low as 20° C between the shocks cellular metabolism in the explants was slowed down and the majority of the cells, prevented from entering mitosis during the heat shock, were not able to complete cell-division during *the following period of sub-optimal temperature either.* Judging from HARRISON and KLEIN's (1954) investigations growth and differentiation in chick blastoderms will proceed at least 30 times more slowly at 20° C than at 37.5° C. However 20° C is by no means an absolute barrier for cell division. This fact *was soon realized in the cytologic control of the explants,* and moreover it was also indicated by the finding that the wave-promoting effect from the treatment was lower than expected. In the lastmentioned type of experiments the treatment usually involved 5—7 × 30 min at the supra-optimal temperature, alternating with 4—6 × 30 min at the sub-optimal temperature and followed by incubation at 37.5° C.

Under such conditions an average mitotic index in the explant of up to 12 per cent could be attained during the following waves of mitoses. In controls the index lay all the time at about 2 per cent.

In Fig. 2 is demonstrated a typical result from an experiment of the type just described. The synchronism which was effected is manifest in the explant still after the completion of two successive cell cycles. In this series and in the others of the same sort the generation-time of the synchronized cells was found to be 12—14 hours, i. e. longer than after a single shock.

Morphogenesis was in no case quite normal during the incubation at optimum temperature after the shocks, but approximately one third of the embryos exhibited less severe disturbances. In the examination of these embryos it was found that the morphologic disturbances *had especially affected* the outgrowing mesoderm. Development of somites, for instance, was only exceptionally observed. Deformation of the neural tube as well as the primary brain vesicles were frequent.

The distribution of the accumulated mitoses was apparently much similar to that observed in embryos which had been exposed to one single shock. In

addition it was now possible to establish more exact values for the accumulation. Even if individual variations in the average accumulation were considerabl in the c. 100 explants investigated, the distributional pattern of the mitoses during the first mitotic wave after the heat shocks was one and the same. Cf. Fig 1 B.

A representative of these explants is shown in Fig 4 E. In that very explant — as in the others — the highest average mitotic indices (8—10 per cent) are found in the immediate vicinity of the node and in adjacent areas on both sides thereof. More towards the fore part of the explant the index is somewhat lower (6—7 per cent) Slightly lower values dominate in the hind part of the explant.

A major part of the explants further displayed accumulation of mitoses in a narrow zone along the whole edge of the preparation.

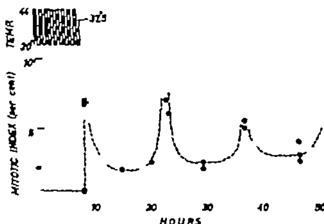


Fig 2. Diagram illustrating the synchronization which is achieved in explanted chick embryos (18-hour-stage) after series of heat shocks. Mitotic indices refer to part of the embryo area, obliquely anterior to the primitive streak. Indices are average values for all cells within the region.

At least for the node region and the area anterior thereto it will seem that the varying accumulation of mitoses rather has its origin in the underlying layers (meso- and entoderm) than in the ectoderm. In the latter layer average accumulation was always strikingly higher than in the two others taken as a whole. In the actual explant, for instance, mitotic indices recorded for the ectoderm amounted to 11—16 per cent against 4—7 per cent for the others. Normal average values for the same stage (in ovo) were found to be ectoderm 4—6 per cent, mesoderm + entoderm c. 2 per cent.

Summarizing the experiences of the heat shocks show that it is possible with supra-optimal temperatures to produce waves of mitoses in the chick embryo, i. to chieve a partial synchronization of the cell material. However to

prevent division of the embryo cells the temperature must be considerably elevated above the optimum (37.5 C). RNA-synthesis is apparently very sensitive to change but probably there is still some margin left before also the DNA-synthesis is affected. Through the compensatory increase of mitoses after a heat shock patterns in cell multiplication, if existent, can be more easily distinguished and by securing the time for the next wave of mitoses an approximate measure at least of the generation time of the cells is obtained. However it is apparent that different types of heat shocks give different values of the latter.

C. Treatment with Mitotic Inhibitors

Now indications of a higher mitotic activity around the anterior part of the primitive streak and in the ectoderm had been clear enough in the heat shock experiments. But owing to the obvious disturbances in chromosome morphology as well as embryo morphology it was not precluded that the observed distribution of the accumulated mitoses actually reflected a varying heat sensitivity of the blastoderm cells.

It was assumed that further conclusive information of the mitotic activity in the blastoderms of the primitive streak stage would be obtained with the aid of mitotic inhibitors at the normal incubation temperature, 37.5 C. The inhibitors selected have been colchicine and oestradiol. As distinguished from conditions during the heat shocks the inhibition is now in both cases an arrest of mitosis in metaphase. Preliminary investigations had proved that still after 6 hours the beginning disorganization of the arrested mitoses presented no difficulties in the estimation of the mitotic index (or stathmo-kinetic index, as it sometimes is called on such occasions).

Of the inhibitors colchicine, as known has been widely used for localization of mitotic activity and cell proliferation in animal tissues. It has also been used for chick embryos, and strong indications of considerably differing generation times among the tissues of the 2-day-old chick embryo were found by OVERTON (1958) after colchicine inhibition.

The inhibiting action of oestradiol on cell-division in vertebrate cells has been described by, among others, v. MÖLLENDORFF (1943) (rabbit fibrocytes) and TÖNDURY and CAGIANUT (1951) (amphibian embryos). Correspondingly inhibition of mitosis in invertebrate cells is described by AGRELL (1954, 1955) for sea urchin embryos. The latter author has emphasized the similarity between the inhibiting action of colchicine and that of oestradiol, and has made use of the last-mentioned substance to reveal a graded mitotic activity within the sea urchin embryo. AGRELL (1960).

Colchicine

In Fig. 3 are collected mitotic indices for chick blastoderms, incubated for various lengths of time on a medium containing colchicine. The values refer

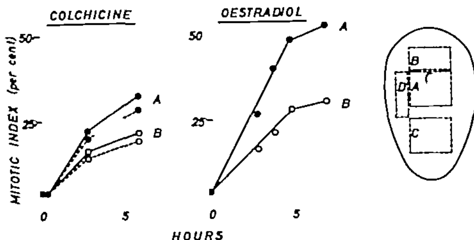


Fig 3. Diagrams showing the accumulation of mitoses produced by colchicine and oestradiol respectively in 18-hour-old chick embryos. Continuous lines refer to explanted embryos, dashed lines to embryos in ovo. The letters A and B stand for regions marked off in the drawing at right. Each point represents an average from three separate embryos.

to two different regions in the blastoderm, regions A and B, which also are outlined in the figure. They comprise A the anterior part of the primitive streak and B the adjacent area in front of the streak. The selection of the particular regions A and B as the principal regions for analysis comes from the fact that they represent adjacent parts of the blastoderm showing the most diverging RNA-concentrations (cf. p. 5) and reasonably ought to be in the best position for showing any differences in mitotic activity if existent. Values are not given for regions C and D in Fig 3 as it appeared that they were intermediate between those of A and B but did not differ significantly from either of them. The reported values are as before mean values for all cells in the delimited region, irrespective of the distribution of the cells among the different germ layers.

As the produced arrest of mitosis was complete, the mitotic figures observed in embryos treated with colchicine for 3 hours and more are almost exclusively metaphases. Of course, there is a small fraction of prophases too, but anaphases and telophases are absent.

The diagram in Fig 3 demonstrates that all through the investigated period accumulation of mitoses is greater in region A than in region B — both in ovo and in vitro. The difference is significant and has to be taken as an indication of a different mitotic activity in the investigated regions. The distribution of the mitoses is apparently the same as after the heat shocks.

It was surprising to find that accumulation was slightly slower in ovo than in vitro, as according to WADDINGTON (1952) the rates of both growth and differentiation of early chick embryos are slower in vitro than in ovo. This may

Table II Mitotic indices (per cent) in primitive streak embryos incubated for 3 hours in vitro on media containing colchicine or oestradiol. Location of the regions A and B is given in Fig 3

		Ectoderm	Mesoderm + Entoderm
Colchicine	A	33.9 ± 1.4	18.1 ± 1.0
	B	31.3 ± 1.4	13.9 ± 0.8
Oestradiol	A	33.3 ± 2.6	21.5 ± 1.3
	B	33.2 ± 2.6	13.2 ± 1.2

be a thermal effect. In spite of all precautions the eggs may have been chilled more than expected during the application of the colchicine.

Earlier observations by the present author have indicated that chick embryos culuvated in vitro on the basic medium alone show practically no decrease in the average mitotic index during the first 10 hours of cultivation. In Fig 3 it appears, however that both in ovo and in vitro the accumulation of cells is taking place at a slightly decreasing rate. It is doubtful whether this is to be considered as the normal condition in the embryo tissue, or if it can be ascribed to a general slackening effect from the colchicine. The latter alternative seems the more probable, as it is known that colchicine is toxic in high dosage, Lustabadoh (1956) However the present concentration of colchicine has scarcely been too high as nuclear damage in the treated embryos is insignificant. Besides the same concentration is not toxic for chick embryos of 50—60 hours incubation (Overton 1958)

The morphology of the treated blastoderms was markedly changed after treatment with colchicine. Thus there had occurred a pronounced shrinkage of the area pellucida (as well as the area opaca) during the treatment, resulting in a reduction of the original width of the area pellucida about two thirds after 3 hours incubation. The shrinking effect from colchicine is manifest also in older chick embryos, causing much distortion of the outgrowing organs. The shrinkage appears to be directly related to changes in cell shape. Investigations by Mazuraki (1949) seems to indicate that colchicine acts upon viscosity of the cytoplasm in animal cells with the effect that the cells adopt a less extended shape than before.

The estimation of the mitotic index in the investigated regions has been combined with inspections of the distribution of the mitoses in order to check whether they are evenly distributed between the germ layers or show a tendency to local accumulation. It appeared from these examinations that for both the regions A and B accumulation of arrested mitoses is much more rapid in the ectoderm than in mesoderm + entoderm. Cf. Table II Further the figures intimate that the difference between A and B has little reference to the ectoderm.

Oestradiol

In the present situation it was of interest to have the found regional differences further confirmed in other similar experiments, based upon a different mitotic inhibitor For this purpose oestradiol was chosen. The author has tested the inhibiting action of pure oestradiol as well as that of oestradiol-benzoate. It appeared that the former had a most decided effect with complete arrest of all mitoses in explanted embryos when present in a concentration 10^{-4} Added in the corresponding molar concentration oestradiol-benzoate arrested only about 20 per cent of the cells entering mitosis.

Attempts to make a direct comparison of embryos treated with oestradiol in vitro and in ovo respectively have failed. Injection of oestradiol in the same concentration as applied in the experiments in vitro did not lead to pronounced accumulation of metaphases in embryos in ovo. Not even after injection of the fourfold concentration was there a complete arrest of any major part of the cells undergoing mitosis, and further increase of the concentration was not tried as already the concomitant increase in the alcohol (solvent!) concentration was not quite harmless to the embryo cells.

In essential respects the results from treatment with oestradiol in vitro, Fig 5 are in agreement with that obtained with colchicine. Also now there is a significant difference in the rate of accumulation of mitoses between the regions A and B and as before accumulation of arrested mitoses in region C and D is intermediate between that in A and B respectively

This time accumulation of arrested metaphases is still taking place at an even rate after 5 hours incubation with the inhibitor In that period roughly 50 per cent of all cells in A are arrested Subsequent accumulation proceeds at a slower rate, presumably as the result of a beginning disappearance of the first arrested mitoses. As a matter of fact, it is rather difficult to present exact mitotic counts in chick embryos treated with oestradiol for more than 7—8 hours. At that stage of the treatment the chromosomes in a great part of the arrested mitoses actually have begun to decompose.

From the intensity of the accumulation it may be concluded that the mean generation time of the cells in region A is about 10 hours, while for the cells in region B it amounts to about 15 hours. Corresponding values after colchicine inhibition are of the same magnitude. Table II further informs that for ectoderm cells the generation time is about 8 hours.

The calculated times are in fairly good agreement with values reported for chick embryo cells in tissue culture (Ris 1955) In the literature no information is given about length of generation time in vivo for embryos just at the primitive streak stage, but for embryos of c. 50 hours' incubation calculated values vary between 8.9 hours (Solomon 1957) and 10.6 hours (Woodard 1948)

Localization of the arrested mitoses is the same after treatment with oestradiol as with colchicine, i. e., accumulation of metaphases is most prominent in

the ectoderm cells, decreasing in the mesoderm and still *more so in the entoderm*. Cf. also Table II

Oestradiol has no shrinking effect upon the preparation. In fact it has instead a *loosening* effect upon chick embryo tissues so that blastoderms treated with it have a tendency to flatten out. Even the arrested metaphases do not appear so contracted as during colchicine inhibition.

Discussion

In the present investigation it has accordingly been possible to demonstrate a higher mitotic activity around the anterior part of the primitive streak than in the section of the blastoderm in front of it. The result was strongly indicated already in the heat shock experiments, but was definitely substantiated only with the aid of mitotic inhibitors. On the whole it confirms DERRICK's (1937) observations, though with accentuation of mesoderm and entoderm as the principal sites for the varying mitotic activity within the actual part of the blastoderm. Of these two again, mesoderm has showed greater heterogeneity with respect to the cell multiplication.

However even now the established difference must be considered as rather moderate, and it will scarcely make the primitive streak stand out as a regular zone of proliferation.

Within the embryo area of the 18-hour-blastoderm there is evidently no region which decisively can be distinguished as such a zone. But if one wishes to emphasize the mutual dependence of cells on different levels in that area, one can, of course, characterize the whole ectoderm as a zone of proliferation in relation to the other germ layers. In investigated normal embryos the relative amount of mitoses was always definitely higher in that layer and this condition was, of course, still more pronounced in those embryos in which an accumulation of arrested mitoses had been produced.

The fact that the observations to a large extent are based upon embryo explants will not invalidate the obtained result. Indeed growth and differentiation are slightly slower in explanted embryos than in embryos in ovo, but as far as known this retardation will affect all cells equally and so the explant is still directly equivalent to the normal embryo. Evidence of a continued maintenance of the normal relation between the cells in the embryo area — even after explantation to a basic culture medium of the kind employed here — is given by the fact that such explants will show normal morphogenesis during their further development in vitro.

One complication when comparing mitotic activities in the various regions of the early blastoderm with the aid of mitotic inhibitors is that the primitive streak is no permanent structure but will last only for a comparatively short time. Partly as a result of cell-streaming the streak will soon regress and eventually disappear. For the interpretation of the observations above it is necessary to know whether one can regard a result — obtained through a successive gathering

of cells entering mitosis — as representative for conditions prevailing just at the primitive streak stage. The fact that the difference is significant already after 3 hours, when using two different inhibitors of mitosis, is strongly indicative that such is the case. Judging from the observations of SPRATT (1947) the cell-streaming is not rapid enough at this stage to interfere seriously with the result over such a short period.

The occurrence of definitely higher mitotic indices around the anterior part of the primitive streak, i. e. a section of the blastoderm with a comparatively low value of the quotient RNA/DNA for the stage in question, is reminiscent of conditions in the more advanced chick embryos, investigated by the author. In these embryos (23—40 hours of incubation) regions with the lowest RNA concentrations are indubitably characterized by a noticeably high mitotic activity.

It is doubtful, however, whether the connection observed in the 18-hour blastoderm is fully equivalent to that prevailing later in development in the outgrowing embryo body. For in view of the obvious differences in RNA-concentration between the investigated regions more striking contrasts in mitotic activity were indeed to be expected. Thus it will seem that of the observed RNA-variation at this stage, a good deal at least cannot be attributed to a non-uniform cell multiplication within the blastoderm. Rather it will reflect the differentiation, taking place within the blastoderm.

As each of the selected pieces of the embryo area (1—4 in Fig. 1) reaches right through the blastoderm there is, by the very presence of outgrowing mesoderm in all of them, a general differentiation going on in the whole area. The particularly high concentration of RNA in the region immediately in front of the node may probably be ascribed to the intense protein synthesis during the beginning differentiation of the notochord and neural plate cells. Perhaps a certain accumulation of RNA will also be connected with the onset of induction within the region in question, as according to BRACHET (1957) transfer of RNA is probably involved in that process.

As a matter of fact, histochemical analyses of the present author for RNA (based upon examination of RNAse-treated embryos and controls, both stained with gallocyanin-chromalum) as well as those of GALLERA and OPRECHT (1948) and LAVARACK (1957) have just indicated that in front of the node mesodermal cells, and still more so ectodermal cells, are very rich in RNA. Even if the cells of the primitive streak obviously have a high content of RNA too, the visual estimation of RNA seems to be consistent with the result of the biochemical analyses, i. e. of a higher average value of RNA for the region immediately in front of the streak.

In this connection the regional differences in carbohydrate metabolism reported to occur in chick blastoderms are worth mentioning. According to SPRATT (1950) the anterior part of the primitive streak (the node) has a conspicuously higher energy requirement of carbohydrate than other regions, whether dif-

ferentiated or undifferentiated within the blastoderm, even if it has not yet been possible to prove this in respiratory measurements. The higher mitotic activity in the node region found in the present investigation seems well consistent with SPRATT's observation. The same author also distinguishes a qualitative difference in carbohydrate metabolism between the heart mesoderm and the neural ectoderm, *growing out in front of the node. Thus the latter would* depend primarily upon oxidative metabolism, the latter upon glycolysis however this interpretation has been seriously questioned by DUFFEY and EBERT (1957)

In the present investigation it has been of great interest to follow *the morphogenetic and cytologic disturbances* which have been provoked in the blastoderm by heat and by mitotic inhibitors.

It was not surprising to find that heat treatment affects the synthesis of RNA in the chick embryo *a similar result is reported for amphibian embryos,* STEINERT (1951) As RNA-protein and RNA presumably play an important role in inductive processes in the developing embryo and as furthermore it has been argued that RNA will contribute to a proper cell contact (BRACHET 1957), it seems that there is a direct connection between this heat effect upon RNA and the recorded morphogenetic disturbances.

When analyzing the influence of elevated incubation temperatures on dividing chick embryo cells, one should try to distinguish between effects on DNA-synthesis and effects on the mechanism of division, *i. e.*, on processes connected with the movements of the chromosomes. Apparently the mitotic disturbances produced in short time experiments by extreme temperatures, high as well as low belong to the last mentioned category (KEMP and JUEL 1931 SPEAR 1928). Thus it is also presumed in the present investigation that the compensatory increase of mitotic activity after a heat shock (the mitotic wave) will indicate that mitosis but not DNA-synthesis, was brought to a standstill during the shock. As in the present case the cells were observed to be arrested in prophase, it seems likely that the heat shock has affected such processes as chromosome spiralization migration of the centrioles and aster formation.

In this connection attention should again be given to the above-mentioned decrease of the RNA-content of the heat-shocked cells.

Evidence for a relation between ribonucleoproteins and the chromosome cycle has been presented by JACOBSON and WEBB (1952) and according to GUTTMAN (1956) the chromosomal ribonucleoprotein possibly controls chromosomal spiralization and despiralization. Experimental removal (by RNAse) of cellular RNA in chick fibroblasts, has in fact been found to result in prophasic *inhibition of mitosis (FIRKET et al* 1955) In meristematic cells, multipolar spindles among other abnormalities were found after influence of the same enzyme (KAUFMAN and DAS 1955)

Thus it appears that a disturbed RNA-synthesis may actually account for both the morphologic and chromosomal disturbances, observed after the heat shocks.

The morphogenetic disturbances in chick embryos, caused by oestradiol are similar to those effected by heat treatment in the respect that the mesoderm is especially affected. Blastoderms explanted at the primitive streak stage to a culture medium, containing a low concentration of oestradiol (2.6 µg/ml medium) show no morphogenesis after 24 hours incubation. When explanted at the 20-hour stage, one observes after the same time a neutral tube and brain vesicles, but heart and somites are missing. If explants of the primitive streak stage are first incubated for half an hour on a medium containing oestradiol (2.6 µg/ml medium) and then are transferred to a normal medium, they will show a slight retardation only in the development of heart and somites. The disturbances will possibly depend on interference with the synthesis of RNA. For the closely related substance stilboestrol there are strong indications of just a blocking effect upon RNA-synthesis in chick embryo cells (RICKENBACHER 1956). In Triton embryos a diminished RNA-content in the cells is found after treatment with oestradiol (TÖNDURY and CAGIANUT 1951).

The rectilinear accumulation of arrested mitoses in the oestradiol-treated chick embryos indicates that there is no blockage of DNA-synthesis during the interval of the inhibition experiments. It is quite a different matter that after prolonged treatment at high oestradiol-concentrations signs of degeneration are observed in the arrested cells, the destruction being accompanied by a transformation of the chromosome material into one or more intensely stained droplets. Experiments in vitro have shown, however, that oestradiol can act as a substrate activator for DNAse by separating the proteins from DNA (AGRELL and PERSSON 1956).

By choosing a sufficiently high concentration of oestradiol in the experiments a total arrest of all mitoses was secured, and the apparent differences in sensitivity of the germ layers towards the concentration of oestradiol, as observed in explants, could be disregarded. The fact that a similar distribution of arrested mitoses between germ layers is found after colchicine inhibition, cf. Table II, is taken as an indication that oestradiol has not specifically changed mitotic activity of any particular germ layer within the investigated region.

Neither is it likely that the established difference in cell multiplication between region A and region B reflects a qualitatively different reaction towards oestradiol. Actually all observations intimate that the regional differences established by means of oestradiol are directly equivalent to those demonstrated in the colchicine experiments.

The real striking difference in action between oestradiol and colchicine is the dissimilar ability of mitose-accumulation that they show when the embryo material has been exposed to them for more than 3 hours. In the experiments the rate of accumulation will thus decrease in the colchicine material, whereas it proceeds at the earlier rate in oestradiol-treated explants. As already suggested, this decreasing rate observed during treatment with colchicine is probably effected by the colchicine itself. For as this inhibitor produces the same



Fig. 4 A. Normal chick embryo at the primitive streak stage (18 hours of incubation).
B. Chick embryo at the primitive streak stage incubated with colchicine in ovo for 3 hours. Notice the mottled appearance, characteristic for embryos exposed to colchicine in experiments of short duration.
C. Chick embryo at the primitive streak stage incubated with colchicine in ovo for 6 hours. The extreme shrinkage of the embryo is very apparent. (Notice the different magnifications in A, B and C.)
D. Explanted chick embryo (anterior half of the embryo area) immediately after a series of heat shocks. Notice the broadened primitive streak.
E. Explanted chick embryo (anterior half of the embryo area), exposed to a series of heat shocks and then incubated for 10 hours at 37.5° C. A developing neural tube is vaguely discerned in the midline of the explant. Incubation was interrupted at the culmination of the second mitotic wave.
F. Explanted chick embryo (anterior half of the embryo area) exposed to a series of heat shocks and then incubated for 20 hours at 37.5° C. Notice the disturbed outgrowth of the neural tube and the absence of somites.
G. Characteristic appearance of prophases of chick embryo cells during incubation at normal temperature (37.5° C) after previous heat shock.
H. Chick embryo cells, arrested in metaphase by colchicine.
I. Chick embryo cells, arrested in metaphase by oestradiol.
Staining: D Giemsa, otherwise Gomori hematoxylin.

accumulation of mitoses in vivo as well as in vitro the decrease does not reflect a mitotic pattern particularly characteristic of explants. Neither is there discernible any stimulating action on the cells from the oestradiol in fact both inhibitors have during the first 3-hour-period given practically the same rate of accumulation and it is only just maintained in the explants treated with oestradiol. Otherwise it has been demonstrated that oestradiol can have a stimulating action on mitotic activity (BULLOUGH 1955)

In view of these observations it seems that oestradiol is preferable to colchicine for determination of cellular doubling times in chick embryo explants. In experiments on chick embryos in ovo colchicine has been the only possible of the two, but care must be taken that the produced accumulation is recorded not later than 3 hours after the onset of the experiment.

The shrinkage of the embryo area after treatment with colchicine emphasizes the fact that besides cell multiplication and cell streaming also changes in cell shape play an important role in accomplishment of a normal morphogenesis. Differences in cell shape are very apparent at the primitive streak stage, the extremities are the tall columnar cells in the ectoderm of the streak region and the flat endodermal cells. Moreover SPRATT (1956, 1958) has recently called attention to the existence in the intact blastoderm of distinct intercellular fibres, which apparently are of great importance for the proper contact between the embryo cells.

The present observations in chick embryos, treated in ovo with colchicine for 1—3 hours (Fig 4 B) suggest an arrangement within the area pellucida of distinct points with particularly intense adhesion between the cells. It seems that colchicine may be a useful tool for estimation of the quantitative distribution of the above-mentioned intercellular filaments.

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References

AGRELL, I Oestradiol and testosterone propionate as mitotic inhibitors during embryogenesis. Nature (Lond.) 1954 *173*, 172.

AGRELL, I L action de l'oestradiol sur les premières phases du développement embryonnaire de l'Oursin Paracentrotus lividus (L) C. R. Soc Biol. 1955, *149* 1322—1327

AGRELL, I Detection of graded mitotic activity within the sea urchin embryo through the use of oestradiol. *Ark. Zool.* 1960, *12*, 411—414.

AGRELL, I and H PERSSON The action of oestradiol on desoxyribonucleoprotein as the cause of its inhibitory effect upon mitosis in the sea urchin embryo. *Biochim. biophys Acta* 1956, *20*, 543—546.

BELLAIRS, R., Studies on the development of the foregut in the chick. III. The role of mitosis. *J Embryol. exp. Morph.* 1955, *3*, 242—250.

BELLAIRS, R., Studies on the development of the foregut in the chick IV Mesodermal induction and mitosis *J Embryol. exp. Morph.* 1957 *5*, 340—350.

Brachet J "Biochemical Cytology" New York 1957
Bullough, W. S., Hormones and mitotic activity Vitam. and Horm. 1955. 13. 261—292.
Derrick, G. E., An analysis of the early development of the chick by means of the mitotic index. J. Morph. 1937 61 257—284
Deuchar, M., The effect of a high temperature shock on early morphogenesis in the chick embryo. J. Anat. (Lond.) 1952. 86. 443—459
Duffey L. M. and J. D. Ebert Metabolic characteristics of the heart-forming areas of the early chick embryo. J. Embryol. exp. Morph. 1957 5. 324—339.
Emanuelsson, H., Variations in nucleic acid concentration during the development of early chick embryos. Acta physiol. scand. 1958. 44 336—364
Firket H., Chèvremont-Comhaire, S. and M. Chèvremont Action of ribonuclease on living cells in vitro and synthesis of deoxyribonucleic acid. Nature (Lond.) 1955. 176. 1075—1076.
Gallera, J. and E. Oprecht Sur la distribution des substances basophiles cytoplasmiques dans le blastoderme de la poule Rev. suisse Zool. 1948. 55. 243—250.
Guttman R., Effects of kinetin on cell division with special reference to initiation and duration of mitosis. Chromosoma (Wien) 1956. 8. 341—350.
Hamburger, V. and H. L. Hamilton A series of normal stages in the development of the chick embryo. J. Morph. 1951 88. 49—73.
Harrison J. R. and I. Klein Effect of lowered incubation temperature on the growth and differentiation of the chick embryo. Biol. Bull. 1954 106. 48—59
Hoffman, J. G., Theory of the mitotic index and its application to tissue growth measurement. Bull. Math. Biophys. 1949. 11 139—144
Jacobson, W. and M. Webb, The two types of nucleoproteins during mitosis. Exp. Cell Res. 1952 3. 163—183
Kaufman, B. P. and N. K. Das, The role of ribonucleoproteins in the production of mitotic abnormalities. Chromosoma (Wien) 1955 7 19—38.
Kemp T. and J. Juul, Der Einfluss der Wärme auf die Zellteilung Arch. exp. Zellforsch. 1931 11 602—617
Lavarack, J. O. The behaviour of basophil cytoplasmic substances during neural induction in the chick. J. Embryol. exp. Morph. 1957 5. 111—121
Leblond, C. C., Morphologic methods of determining cellular doubling times J. Histochem. Cytochem. 1956. 4 499—507
Melander, Y. and K. G. Wingstrand, Gomori hematoxylin as a chromosome stain. Stain Technol. 1953. 28. 217—223
Mitchell, B. Effects of colchicine on resting cells in tissue cultures Exp. Cell Res. 1949 Suppl. 1. 420—431
v. Möllendorff W. Zur Kenntnis der Mitose XII. Z. Zellforsch. 1943 32 33—82.
Overton J. Effects of colchicine on the early chick blastoderm. J. exp. Zool. 1958. 139 329—347
Pasteels, J. Prolifération et croissance dans la gastrulation et la formation de la queue des vertébrés. Arch. Biol. (Paris) 1943. 54 1—51
Riedelsbacher, J. Die Wirkung des Stilboestrols auf die Keimscheibe des Huhnchens. Z. Zellforsch. 1956. 45. 339—377
Rie, H., In Analysis of Development Ed. by B. H. Willier, P. Weiss and V. Hamburger Philadelphia & London 1955 91—125
Solomon, J. B., Increase of deoxyribonucleic acid and cell number during morphogenesis of the early chick embryo. Biochim. Biophys. Acta. 1957 23. 24—27
Spear, F. G., The effect of low temperature on mitosis in vitro. Arch. exp. Zellforsch. 1929 7 484—492.
Spratt N. T. Jr. Regression and shortening of the primitive streak in the explanted chick blastoderm. J. exp. Zool. 1947 104 69—100.

Spratt, N. T. Jr., Nutritional requirements of the early chick embryo. III. The metabolic basis of morphogenesis and differentiation as revealed by the use of inhibitors. *Biol. Bull.* 1950. *99* 120—135.

Spratt N. T., Jr., Metabolism of the early embryo. *Ann. N. Y. Acad. Sci.* 1952. *55.* 40—50

Spratt N. T., Jr., Form changes in the early chick blastoderm and the probable role of inter cellular fibers. *Anat. Rec.* 1956. *124* 364—365.

Spratt N. T., Jr., in "The Chemical Basis of Development" Ed. by W. D. McElroy and B. Glass. Baltimore 1958. 629—646.

Steinert, M., La synthèse de l'acide ribonucléique au cours de développement embryonnaire des batraciens. *Bull. Soc. Chim. Biol.* (Paris) 1951 33. 549—554

Törecky G. and B. Csabafut Zur Wirkung der Sexualhormone auf Wachstum und Differenzierung. *Biol. Rev.* 1951. 26. 28—38.

Waddington, C. H., "The Epigenetics of Birds" *Cambridge* 1952.

Walker, P. M. B., The mitotic index and interphase processes. *J. exp. Biol.* 1954. *31* 8—15.

Woodard, T. M., The mitotic index in the chick embryo. *Amer. Naturalist* 1948. *82* 129—136.

Zeuthen, E. and O. Scherbaum, Synchronous divisions in mass cultures of the ciliate protozoan Tetrahymena pyriformis, as induced by temperature changes. *Colston Papers* 1954. VII. 140—156.

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Effect of Intravenous Infusion of Glucose on Gastric Secretory Responses to Feeding in Pavlov- and Heidenhain-pouch Dogs

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Abstract

DOTEVALL, G. and A. MUREN. *Effect of intravenous infusion of glucose on gastric secretory responses to feeding in Pavlov- and Heidenhain-pouch dogs.* Acta physiol. scand. 1961. 52. 234—241. — Glucose solution was infused intravenously (30 %, 80—100 ml, 30 min) in conscious dogs with Pavlov- or Heidenhain-pouches during secretion elicited by different procedures. In Pavlov-pouch dogs pronounced inhibition of the secretory response was observed during sham-feeding or teasing with food; the response to feeding was also temporarily depressed, at least during the first two hours after feeding. In Heidenhain-dogs the response to feeding compared with the Pavlov-dogs was not significantly affected except during special conditions. It is suggested that the induced hyperglycemia mainly depresses the nervous phase of secretion, possibly both at the central and the peripheral level.

The stimulating effect of hypoglycemia on gastric secretion has been extensively studied and the insulin test is commonly used as a way of judging whether the vagal innervation is intact or not. The effect of hyperglycemia on gastric secretion, on the other hand, has not attracted much attention. There are, however, some reports on an inhibitory effect on secretion obtained by acute administration of glucose (OKADA *et al.* 1934, NOBLE and ROBERTSON 1938, DAY and KOMAROV 1939, FRIEDMAN 1939, SOLOMON and SPIRO 1959). In most of these cases very large doses of glucose were given. The mechanism of the inhibition has not been further analysed, but BABKIN (1950) has discussed the different possibilities and he suggests that hyperglycemia induced by intra-

venous administration of glucose may lower the irritability of the vagal secretory center in the brain, just as hypoglycemia raises it. Others believe that the secretory inhibition caused by hyperglycemia is largely a peripheral osmotic effect (NOBLE and ROBERTSON, DAY and KOMAROV FRIEDMAN)

In patients with diabetes mellitus, a more or less permanent hyperglycemia is often observed, in spite of treatment with insulin. It has been found that the incidence of duodenal ulcer as well as the gastric secretory activity is reduced in patients with diabetes mellitus (for literature see DOTEVALL 1959 1961) Furthermore there seems to be an inverse relationship between the degree of hyperglycemia and the secretory capacity of the stomach (DOTEVALL 1961) Experiments on dogs made diabetic by treatment with alloxan or by pancreatectomy (MAYO *et al.* 1958 BARCENA *et al.* 1956 SOELZER 1960) have, on the other hand given results which are not in accordance with those mentioned above. Such experiments do, however involve so complex changes with influences on different mechanisms that it is difficult to draw any conclusions from these results, — these reservations should be borne in mind even in the cases of human diabetes. There are thus reasons for believing that hyperglycemia may directly or indirectly at least to some extent be responsible for changes in gastric secretory activity

The present paper is concerned with the effect of hyperglycemia on gastric secretion after feeding and other stimulating procedures in Pavlov and Heidenhain-pouch dogs. A preliminary report of the result has been given previously (DOTEVALL and MUREN 1960)

Material and methods

The experiments were made on 10 dogs weighing 12—15 kg Pavlov- or Heidenhain-pouches had previously been made In some dogs the Pavlov-pouch was changed into Heidenhain-pouch after period of time so that experiments were made on the same dog with both types of pouches. Oesophageal fistulae were made on two dogs.

Feeding was controlled throughout the experiments. The dogs were given water and standard dog food (Bonzo) consisting of ground and dried meat, fish and bread formed into small cubes. The experiments were performed on conscious dogs who had been fasted during 24 hours. Before the test meal was given, consisting of 200 g of this food, a needle was introduced into vein of one hind leg The needle was connected to an infusion pump via polythene tube. Infusion of 30 % glucose solution was given usually during 30 min and at rate of 3 ml/min (i.e. total of 2.5 g glucose per kg body weight). The gastric juice was collected in 15 min periods. The amount of total acid was determined by titrating each portion against N/10 NaOH with phenolphtalein as an indicator

Blood sugar was determined in some of the experiments according to Somogyi-Nelson's method (NELSON 1944)

Results

1 *Pavlov-pouch dogs.* Nine experiments were made on four dogs. Glucose infusion was started 30—90 min after feeding or during the last part of the rising phase of the secretion curve. In all experiments this caused a distinct depression of

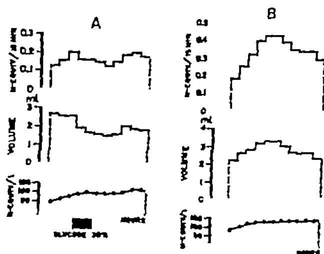


Fig. 1 A. Mean of all observations with glucose infusion after feeding in Pavlov-pouch dogs.

B. Mean of all control experiments after feeding in four Pavlov-pouch dogs.

the secretion, measured in milliequivalents per period in most cases both volume and acidity were reduced. The effect of infusion was considered an inhibitory one, when the last sample during infusion and at least the two following ones had lower values than the one preceding and two succeeding samples. The mean of all these observations is given in Fig 1 A. The mean of 7 control experiments after feeding only is given in Fig 1 B Fig 2 presents a comparison of the effects in one and the same dog with high secretion of 30 % glucose, isotonic glucose and the response to feeding only Fig 5 A presents the effect of 30 % glucose in a dog with very low secretion. In most cases the effect of the glucose on secretion did not become noticeable until 15 min after starting the infusion. The maximal depression occurred during the first or second period after infusion. The secretion was never completely abolished,

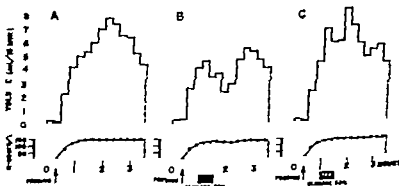


Fig. 2. Three experiments on one and the same Pavlov-pouch dog (2—3 weeks between each exp.)
A. Feeding only B. Feeding with infusion of 30 % glucose. C. Feeding with infusion of isotonic glucose.

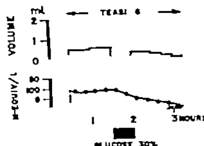


Fig 3. Glucose infusion during teasing in Pavlov-pouch dog.

only more o less reduced, usually for 45 min after the end of infusion. The infusion of 30 % glucose caused a bloodsugar increase up to 300—400 mg % whereas with isotonic glucose solution, the level of bloodsugar did not exceed 150 mg % which had little or no effect on gastric secretion.

A general observation made during the experiments was that the inhibitory effect of glucose was most pronounced during the early phase of secretion. When glucose was administered more than two hours after the onset of secretion, its inhibitory effect was insignificant or it did not appear at all.

2. *Sham-feeding or teasing* These procedures, which were tried in 6 experiments on 5 dogs, gave rise to a relatively small and somewhat variable secretion but the reaction to glucose was very striking The infusion of 30 glucose solution caused a rapid and pronounced depression of the secretion (Fig 3) In most cases the secretion was practically abolished during quite a long period of time after infusion.

3 *Heidenhain-pouch dogs* In 13 experiments on 4 different dogs glucose was infused during secretion after feeding using the method as previously described for Pavlov-pouch dogs. In these cases the secretory response to feeding was, as might be expected, smaller In 7 out of these experiments no effect on secretion could be observed (Fig 4 5 B) In 4 experiments a minute lowering of volume was seen 45 to 60 min after the end of glucose infusion, in one of those also a slight lowering of acidity In extent these effects were however so small that they in no way could be compared with those regularly found in the Pavlov-pouch dogs. In the remaining 2 experiments a slight but definite inhibition was observed however in these cases there was a basal secretion of acid before feeding This secretion was not due to an incomplete fasting but the experiments were made only 3 weeks after changing the previous Pavlov-pouch into a Heidenhain-pouch, a procedure which sometimes has been found to give rise to a basal secretion for which the cause is so far not known.

Control experiments a) Insulin, 0.5 IU/kg subcutaneously was given in 8 experiments on Pavlov-pouch dogs. The secretion started within one hour Infusion of 30 glucose brought about an abolition of the secretion which lasted for about one hour (Fig. 6) There was a good correspondence between the bloodsugar level and the secretory rate. The fall in secretion started within

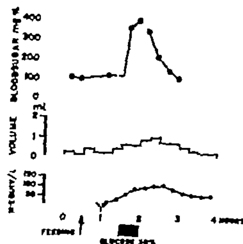


Fig. 4. Glucose infusion during secretion after feeding in Heidenhain-pouch dog.

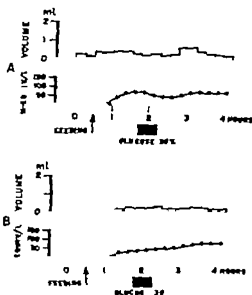


Fig. 5. *Glucose infusion during secretion after feeding in one and the same dog.*

A. The dog is equipped with a Pavlov-pouch.

B. Three months after changing the Pavlov-pouch into Heidenhain-pouch.

the first minutes of the infusion, during which the bloodsugar increased up to 300—400 mg %. During the falling phase of the bloodsugar level the secretion did not reappear until the level of blood sugar had reached 60—50 mg %.

b) Saline solution isotonic (0.9 %) as well as hypertonic (5.0 %) was infused in several experiments during a secretion phase caused by insulin or by feeding. No effects of these infusions were observed, neither on volume nor acidity (Fig. 7).

c) Isotonic glucose solution did, as previously mentioned, not significantly influence the secretion, but in the amounts given it raised the bloodsugar level only to about 150 mg %.



Fig. 6. Effect of glucose infusion on the secretory response to insulin.



Fig. 7. Infusion of hypertonic saline solution during insulin stimulated secretion.

Discussion

The present results confirm previous findings of an inhibitory effect on gastric secretion caused by intravenously administered glucose. As for the mechanism responsible for this inhibition, the results indicate that hyperglycemia mainly affects the nervous phase of secretion. A peripheral, inhibitory effect due to the increase in osmotic pressure of the blood may be ruled out in the present experiments as saline solutions of the same osmolarity as th glucose solution used did not affect any type of secretion. This is not in accordance with the opinion of the previously mentioned uthors, who have made the increase in *osmotic pressure responsible for the peripheral inhibition of secretion. This* discrepancy may very well be explained by the differences in dosage. In most previous studies large doses of glucose were given, or the solutions were injected intravenously during a short period of time, as compared with the slow intravenous infusions performed in the present experiments.

Even if the effect of hyperglycemia may be essentially restricted to the nervous phase of secretion there are still a number of different locations and ways through which the effect may operate.

a) Cerebral structures, including cortical areas, vagal secretory centers and synaptic connections may very well be affected at different levels. Since the dogs in the present experiments were conscious, it is possible that the induced hyperglycemia depresses vagal activity by temporarily abolishing the feeling of hunger and appetite. This could very well explain the rapid inhibition of the secretion induced by sham-feeding or by teasing. As for the different subcortical structures, there is very little information at present available, but it has been assumed by several authors that the vagal secretory center is inhibited by hyperglycemia. The present studies do not allow any conclusion to be drawn regarding the central effect of hyperglycemia. It is possible that some information could be obtained by using different types and levels of anesthesia. In this connection it should be borne in mind that a possible, central inhibition may be due either to a depression of the basal activity of vagal centers or by activation of inhibitory fibres, the existence of which is yet uncertain.

b) Peripheral synapses, i. e. the connection between central vagal fibres and intramural neurons and between the different neurons constituting the nervous plexa in the stomach walls, may be inhibited by hyperglycemia. From the neurophysiological point of view no investigations seem to have been made on the effect of hyperglycemia on synaptic impulse transmission. The present results do not offer any direct evidence in favour of an inhibitory effect of glucose operating at this level. However recent experiments, partly on the same dogs, suggest that such a mechanism may be engaged, as the effect of carbachol, mainly exerting its action on the synaptic level, is also inhibited by glucose infusion.

c) Nerve endings or neuroeffector junctions may be influenced by glucose. The above mentioned recent study indicates that intravenous infusion of glucose to some extent inhibits the secretory responses not only to carbachol but also to mecholyl and urecholine while the histamine effect is quite unaffected (DOTEVALL and MURBY 1961). This may mean that glucose selectively interferes also with the stimulation process at this level. It is generally assumed that histamine exerts its action very close to the secretory cell. However since so little is known about the physiological mechanism operating at this point no further conclusions can at present be drawn about these problems.

It may be concluded from the present results that acutely induced hyperglycemia can inhibit gastric secretion. There is reason to believe that it is mainly the nervous phase which is influenced and the effect may at least in part, be localized at a peripheral level. The experiments give no evidence in favour of the assumption that the increase in osmotic pressure induced by the glucose administered inhibits the activity of the secretory cells directly.

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References

BABKIN, B. P., Secretory mechanism of the digestive glands. *Hoeber* New York, 1950. pp. 631—657

BARCENA, J. J., L. BRAVO, C. M. BACON, C. F. MOUNTAIN and L. R. DRAGSTEDT Effects of total pancreatectomy on gastric secretion. *Surg. Forum* 1956. *7* 380—382

DAY, J. J. and S. A. KOMAROV Glucose and gastric secretion. *Amer. J. dig. Dis.* 1939. 6. 169—175.

DOTEVALL, G., Incidence of peptic ulcer in diabetes mellitus. *Acta med. scand.* 1959. *164* 463—477

DOTEVALL, G., Gastric secretion of acid in diabetes mellitus during basal conditions and after maximal histamine stimulation. *Acta med. scand.* In press.

DOTEVALL, G. and A. MUREN, Evidence on selective inhibition of vagally induced gastric secretion caused by hyperglycaemia. *J. Physiol.* (Lond.) 1960. *154* 4—5 P

DOTEVALL, G. and A. MUREN, Effect of intravenous infusion of glucose on gastric secretory responses to different chemical agents. To be published.

FRIEDMAN, M. H. F. The influence of glucose administration on gastric secretion. *Amer. J. Physiol.* 1939. *126.* 493 P

MAYO, JR. H. W. and D. M. EHRMANN, Endocrine influences of the pancreas on gastric secretion. I. The effects of glucagon and alloxan on Heidenhain pouch secretion. *Surgery* 1958. *44* 81—97

NELSON, N., A photometric adoption of the Somogyi method for determination of glucose. *J. biol. Chem.* 1944. *153.* 375—380.

NOBLE, R. L. and J. D. ROBERTSON, The effect of hypertonic solutions on gastric secretion and intraocular pressure. *J. Physiol.* (Lond.) 1938. *93.* 430—437

OKADA, S., S. AOYAMA and A. SCOTTA, Blutzuckerspiegel und Magentätigkeit. *Ztschr. klin. Med.* 1934—35. *127* 89—93.

SOLLER, R. E. The effects of total pancreatectomy on gastric secretion. *Surg. Forum* 1960. *10.* 167—170.

SOLOMON, S. P. and H. M. SPIRO, The effects of glucagon and glucose on the human stomach. *Amer. J. dig. Dis.* 1959. *4* 775—786.

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Effects of Ethanol on the Membrane Potential and Membrane Resistance of Frog Muscle Fibres

By

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Abstract

KNUTSSON, E., *Effects of ethanol on the membrane potential and membrane resistance of frog muscle fibres.* Acta physiol. scand. 1961. 52. 242—253. — The effects of ethanol on the membrane potential and membrane resistance of frog muscle fibres in situ were studied by means of an intracellular microelectrode technique. By continuous recording of the membrane potential level from one fibre as well as by consecutive recordings from different fibres, the time course of the potential changes induced by different concentrations of ethanol was followed for periods of up to one hour. At the lowest concentrations used, 0.05 and 0.1 M, a depolarization, although slow and sometimes irregular, could usually be demonstrated. At concentration equal to or higher than 0.2 M, marked lowering of the membrane potential value was regularly observed. The depolarization rate increased with higher ethanol content, a typical result being a 15 per cent reduction of the initial membrane potential value after 15 min of exposure to 1.0 M ethanol. Reversal of the membrane potential changes after the ethanol had been washed out from the muscle bath was also demonstrated. Determinations of the voltage-current relation of the muscle fibre, either by square pulse analysis or by direct recording of voltage-current curves, revealed a gradual lowering of the membrane resistance, the effect increasing with the ethanol concentration from 0.2 to 1.0 M. At lower concentrations the changes in membrane resistance were within the normal variations found in untreated muscle.

One of the earliest observations on excitability changes induced by ethanol was made by VON HUMBOLDT (1797) who on frog nerve muscle preparations found an initial increase in excitability followed by a decrease leading to final

block. Later BIEDERMANN (1881) by measuring the thresholds for electrical stimulation of motor nerve exposed to ethanol, demonstrated an initial decrease in threshold followed by an increase and similar excitability changes were also found in experiments with direct stimulation of frog muscle exposed to ethanol (BLUMENTHAL 1896).

While numerous studies of the effects of ethanol on the excitability of nerve and muscle tissue have since been published (cf. e. g. LUCAS 1913, BLUME 1925, KOCHMANN 1936) there seems to be but little work bearing on the fundamental membrane processes involved. In a study on frog sciatic nerve, GALLEGO (1948) showed that ethanol applied to a nerve segment caused demarcation potentials indicating a depolarization of the nerve membranes in the affected region. The lowering of the membrane potential was considered as a sufficient explanation for both the increase and decrease in excitability, the latter appearing as a result of cathodal depression at higher degrees of depolarization.

The intracellular microelectrode technique, although applied in numerous studies of other drugs, has apparently not been used in any systematic analysis of the mechanism of ethanol action on excitable membranes. The present paper gives an account of investigations designed to study, with this technique, both the membrane potential level and the membrane resistance in frog muscle preparations exposed to ethanol in different concentrations.

Methods

Preparation. The experiments were done on sartorius muscles of frog (*Rana temporaria*) which were dissected out and placed in a bath containing Ringer solution of the following composition (mM) viz. NaCl 115, KCl 2.5, $CaCl_2$ 1.8, Na_2HPO_4 2.15, NaH_2PO_4 0.85 and with a pH value between 7.0 and 7.5. The whole bath was enclosed in a gas chamber of perspex through which 100 per cent oxygen was passed so as to obtain a constant oxygenation of the bath. In some experiments the bath was oxygenated by bubbling of oxygen through the Ringer solution. For the purpose of studying ethanol action under anoxic conditions, the oxygen could be replaced by nitrogen.

Ethanol was added to the Ringer's solution in the bath. In some experiments the frog's second sartorius muscle was used as control. It was then placed in a separate bath and treated in the same way as the test muscle except that no ethanol was administered. All experiments were performed at room temperatures between 22 and 25° C.

Microelectrodes. The capillary electrodes (tip diameter less than 1 μ) were produced, by means of a pulling device, from Pyrex glass tubes of about 0.8 mm outer and about 0.5 mm inner diameter. The micropipettes were filled with 2.7 M KCl solution by boiling in ethanol at reduced pressure (cf. TASAKI *et al.* 1954). The resistance of the microelectrodes was checked by application of alternating current of constant amplitude across the electrode from a variable frequency oscillator (cf. HAAPANEN *et al.* 1958). For the experiments were chosen electrodes with resistances between 12 and 50 megohms.

Variations in the tip junction potentials of the micropipettes may introduce a certain inaccuracy in the determination of the numeric value of the membrane potential (cf. ADRIAN 1956). To reduce this source of error electrodes with relatively low tip potentials should preferably be used. The tip junction potentials have been measured in the follow-

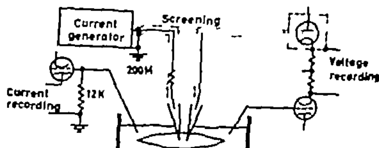


Fig. 1 Diagram of experimental arrangement for determination of voltage-current relation.

ing way The micropipette was first placed with the tip in 2.7 M KCl solution. This solution being of the same composition as the electrolyte within the capillary no potential should develop across the tip junction. The electrode was then transferred to Ringer sol tion and the change in potential level was measured. This corresponds to the tip junction potential, the potential changes at the reference agar Ringer electrode being negligible. For the lectrodes used in the present investigation the tip junction potentials were found to be p to 8 mV The administration to the Ringer' solution of ethanol in the concentrations used in these experiments did not result in any recordable changes of the tip potential.

Membrane potential recording The microelectrod was connected to an input stage of cathode follower type (HAAPANEN and OTTOSON 1954) coupled to dc amplifier (HAAPANEN 1955) driving one channel of a double-beam oscilloscope as well as an inkwriter (frequency esponse 1/sec)

Two different types of experiment were used for the studies of the membrane potential changes. In the first, the resting potential of one fibre was recorded continuously for a comparatively long period, up to bout an hour For such long-lasting recordings a baseline drift has to be anticipated, mainly due to diffusion of ions at the site of the microelectrod (dc drift in the amplifier about 0.5 mV per hour is negligible, see HAAPANEN 1955) The baseline drift was compensated for by measuring the membrane potentials on the inkwriter curve from the baseline obtained by connecting the zero levels registered by th microelectrode in Ringer's sol tion before and after the muscle recording In the second type of experiment, consecutive brief recordings (up to one minute) were made of the membrane potentials of number of different fibres in the muscle before and after administration of ethanol in some cases also after the ethanol had been washed out.

Determination f oltage-current relation. By inserting two microelectrodes into the same cell, usin g one to pass an electric current through the cell and the other to record the resultant potential deflexion, t is possible to study the oltage-current relation, for determination of the membrane resistance (cf FATT and KATZ 1951) The arrangement for the present investigation is shown in Fig 1 The electrodes were kept less than 0.1 mm apart, except in few experiments where the interelectrode distance was 0.4 mm.

Two different procedures were used. In the first, current was pplied as hyperpolarizing square pulses of 50—100 msec duration, given at about two-minute intervals. The applied current was recorded as potential drop across a known resistance on one beam of the oscilloscope and the resultant electrotonic potential on the other beam. The voltage-current elation (V I) was determined when the electrotonic potential had reached a steady level. Ethanol was not added to the bath until several consecutive recordings had shown a reasonably constant V I ratio.

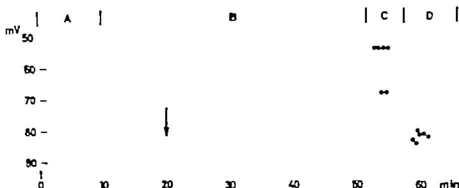


Fig. 2. Ethanol effects on membrane potentials of frog sartorius fibres measured in continuous recording from one fibre (*B*). Ethanol added at arrow concentration 1.0 M. Curve based on values measured from inkwriter curve at one-minute intervals. *A* and *C* control measurements from different fibres of same muscle at beginning and end of experiment. *D* comparative measurements on other sartorius muscle of same frog, not exposed to ethanol. Abscissae time in minutes from onset of recording. Ordinates membrane potential values (minus sign omitted).

In the second procedure, the X-Y display feature of the oscilloscope (Tektronix 502) was used and variations of applied current intensity recorded as vertical deflexions and concomitant changes in membrane potential as horizontal deflexions of the oscilloscope beam. The current generator was operated manually so as to give gradually increasing current from 0.2—0.4 μA inward to 0.1—0.3 μA outward current thus direct recording of voltage-current curves was obtained (cf. FURSHPAN and POTTER 1959 HUTTER and PADSHA 1959) The rate of the gradual increase could be kept rather constant, the average time used for each of these recordings being about 1 sec. For analysis the oscilloscope trace was photographed and its image enlarged and traced onto coordinate papers.

Results

I Changes in membrane potential level

Typical results from an experiment with continuous membrane potential measurement are illustrated in Fig 2. The two sartorius muscles of a frog were placed in separate baths, one of them for exposure to ethanol, the other serving as control First about 10 fibres were measured in order to gain an idea of the average value of the membrane potentials (*A*) the range of which was found to be from 77 to 86 mV with a mean value of 82 mV Then one fibre (*B*) with a membrane potential value in the upper part of this range (84 mV) was selected for the continuous recording During the 10 minutes of recording before ethanol was administered the potential varied between 84 and 89 mV at the moment of administration it was 86 mV

After ethanol had been added to the bath, to a concentration of 1.0 M a gradual depolarization of the membrane could be observed, in this case at a slow rate during the first minutes of ethanol exposure and faster after the 6th minute. In the 22nd to 25th minutes maximum depolarization was reached,

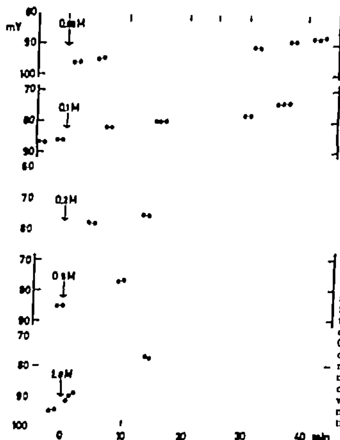


Fig. 3 Continuous recordings of membrane potentials from muscle fibres exposed to ethanol (at arrows) in concentrations of 0 03, 0.1 0.2 0.5 and 1.0 M. Curves obtained from different muscles and based on values measured from inkwriter curve at one-minute intervals. Time in minutes from moment of ethanol administration.

and in the last minutes of the recording the level was comparatively even, the final value after 30 minutes of ethanol exposure being 65 mV

After this continuous recording from one fibre, the membrane potentials of a number of other fibres were also studied (Fig 2 *C*) Measurements of twelve fibres — thus tested after being exposed to ethanol for 35—36 min — gave a mean value of 58 mV range from 52 to 68 mV Fibre membranes not exposed to long lasting microelectrode injury were thus depolarized to about the same extent as a fibre membrane during continuous recording Measurements from the other sartorius muscle of the same frog (*D*) gave a mean value of 82 mV range from 76 to 84 mV The conformity thus found between the records in *A* and *D* indicates that there has been no significant change in the recording conditions nor in the state of the untreated muscle fibre.

Although there may be some variations in different experiments, the depolarization course shown in Fig 2 is characteristic of the effects of ethanol at the concentration 1 0 M in the presence of oxygen. To some extent the depolarization seems to depend on the supply of oxygen in the bath, the depolarization

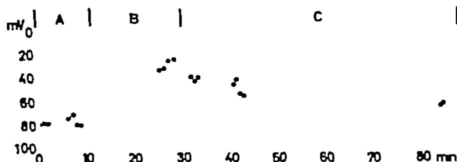


Fig. 4. Ethanol effects on membrane potentials as measured in recordings from different fibres chosen at random. *A* before, *B* after administration of ethanol (concentration 1.2 M). *C* after removal of ethanol. Time in minutes from onset of recording.

rate being considerably slowed down under conditions of anoxia. The difference thus observed may however have a complex background, and it has been deemed necessary to limit the present investigation to experiments with oxygenated Ringer solution.

Continuous recordings of the membrane potentials have been performed on 22 muscles. Fig. 5 illustrates five recordings of this type from experiments at different ethanol concentrations from 0.05 to 1.0 M. At the lowest concentration the depolarization developed very slowly amounting to only 6 mV after 43 min of ethanol exposure. At the higher concentrations applied, the depolarization rate increased with the ethanol content, reaching 17 mV in 15 min at a concentration of 1.0 M. It was fairly characteristic that the depolarization at lower concentrations had an uneven course with fluctuations and "plateaus" whereas at higher concentrations the course was smoother.

A definite depolarization could always be observed when the ethanol concentration was equal to or higher than 0.2 M. At the lower concentrations, 0.05 and 0.1 M, slow depolarizations were usually observed — as shown in the figure — but there have also been cases when no change of the membrane potential was perceptible, as also cases of transient hyperpolarization. These variations may possibly be due to differences in the state of the muscle. Even at a high concentration the rate of depolarization may vary from one experiment to the other. Thus, *e. g.*, in three experiments using a concentration of 1.0 M the membrane was depolarized after 15 min of ethanol exposure to levels that were 12, 15 and 18 per cent, respectively below the initial membrane potential values.

The other method used for the studies of membrane potential changes implied measurements from different muscle fibres in rapid succession during the whole course of ethanol action. This experimental procedure was chosen in order to provide further evidence that the depolarization is not due to a combined effect of ethanol and the injury gradually inflicted to the cell

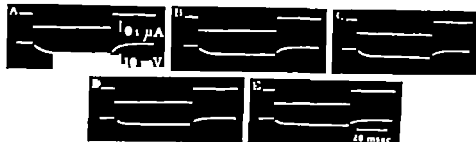


Fig. 5. Ethanol effects on voltage-current relation (V—I) as determined by square pulse analysis. *A* immediately before, *B—E* 5, 10, 15 and 20 minutes, respectively, after administration of ethanol (concentration 1.0 M). Inward current pulses on upper trace, changes of membrane potential (hyperpolarization) on lower trace of each pair. Calibrations in *A* and time bar in *E* apply to all records. Internal electrodes 0.4 mm apart. Membrane potential at start of experiment 88 mV, after ethanol exposure for 20 minutes 66 mV.

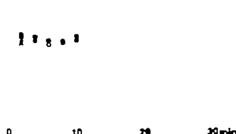
during prolonged microelectrode recording. An experiment of this kind is illustrated in Fig. 4. Measurements of nine fibres show a range of the recording values before ethanol administration between 70 and 85 mV, most of the values being about 80 mV (*A*). After adding ethanol to the bath up to a concentration of 1.2 M (*B*) the membrane potentials were successively reduced to values around 40 mV after about 15 min. Eighteen minutes after ethanol administration the drug was washed out, the muscle soaked in Ringer's solution, and then other cells were tested (*C*). A gradual rise of the values could then be observed. About 50 min after the drug had been washed out, the values of the recordings were between 60 and 80 mV, the highest values thus observed being comparable to the membrane potential values before ethanol administration.

In a similar experiment, using a lower concentration, 0.1 M, a depolarization occurred from 72—80 mV to 50—63 mV during exposure to ethanol for 30 min. At a high concentration, 1.7 M, the depolarization seemed to be of almost the same order of magnitude as at 1.2 M, whereas the repolarization of the membranes after removal of the ethanol was significantly slower.

II. Membrane resistance changes

In order to study the changes in membrane resistance caused by ethanol, the voltage-current relation has been determined by inserting two microelectrodes into the same cell, one to pass current, the other to record the voltage changes induced (cf. Methods). The electrodes have been kept in the same position throughout the experiment, an arrangement which makes it possible to study relative changes of the effective resistance of the muscle fibre but does not give the data necessary to calculate the numeric value of the transverse resistance of the membrane; for this purpose measurements have to be made at varying electrode distances. As, however, certain errors are introduced into the measurements when a muscle fibre exposed to ethanol is subjected to repeated penetrations, determinations of the numeric value of the transverse resistance

Fig. 6 Diagrammatic representation of ethanol effects on voltage-current relation (V I) determined as in Fig. 5. V I ratio in per cent of mean value before administration of ethanol [concentrations 0.1 M ( ), 0.2 M ( ) and 0.5 M (x)]. Internal electrodes less than 0.1 mm apart in all three experiments. Time in minutes from moment of ethanol administration.



have not been included in the present investigation. To what extent the changes observed in voltage-current relation may be referred to changes in the transverse resistance will, however be discussed below

Fig 5 shows records from an experiment using hyperpolarizing square pulses of 0.1 μA and a duration of 50 msec. *A*, recorded before ethanol administration, shows a membrane potential change of 10 mV after the stable level of the electrotonic potential had been reached. The relation between this potential change and the current applied is 10^5 (*Ω*) *B—E* are records made 5 10 15 and 20 min, respectively after adding ethanol to the muscle bath. The stable potential changes were 8, 7 6 and 5 mV respectively revealing a gradual decrease of the V I ratio to 50 per cent of its initial valu after ethanol exposure for 20 min.

In Fig. 6 the results from three typical experiments of this kind have been diagrammatically represented. The ethanol content in the different experiments was 0 1 0.2 and 0.5 M respectively One record was made about every 2 min and the relation V I was estimated. During exposure to ethanol in the concentration 0.5 M the V I raio gradually decreased and after 30 min amounted to 72 per cent of the initial value. At a concentration of 0.2 M there was a slower decline of the V I ratio, i.e. by about 12 per cent in 30 min, whereas in experiments using 0.1 M the declin during that time was 6 per cent which corresponds to the range of variations observed in measurements on muscle in Ringer's solution for the same period of time. A greater change in the V I ratio at higher ethanol content was typical of all experiments (cf. Fig 5) and was most clearly seen when the ethanol concentration was increased stepwise in one and the same experiment.

The second way of measuring changes in the V I ratio, using gradually increasing current (see Methods) *corresponds to the determinations by means of* a whole series of positive and negative square pulse stimulations of different amplitudes provided that the continuous increase of the current is sufficiently slow The time 1 sec for increase from — 0.2 μA to 0.2 μA proved to be slow enough, giving the same V I relation as by application of a series of current pulses. *Fig 7 shows an experiment of this type in which* the current has been gradually changed from about — 0.2 μA to about 0.2 μA, resulting in a concomitant change from 20 mV hyperpolarization to 20 mV depolarization. The

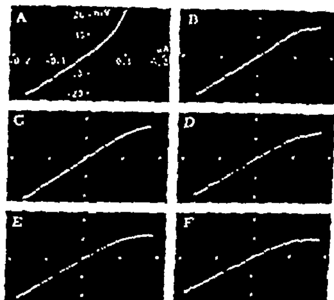


Fig. 7. Direct recording of voltage-current curves obtained before (*A*) and after exposure to ethanol for 5 (*B*), 10 (*C*), 15 (*D*), 20 (*E*) and 25 (*F*) minutes. Oscilloscope spot tracing change in membrane potential (vertical deflexion) as function of polarizing current (horizontal deflexion). Gradual increase of polarizing current in the course of about 1 sec. Inward currents and hyperpolarization of membrane in lower left-hand quadrants, outward currents and depolarization in upper right-hand quadrants. Ethanol concentration 0.5 M. Internal electrodes 0.06 mm apart. Calibrations in *A* apply to all records.

slope of each curve is constant, except when the membrane is depolarized more than 10 mV. After ethanol administration there is a decrease in the linear part of the slope, implying a reduction of the ratio V/I in agreement with the results obtained by square pulse analysis. There also occurs a progressive flattening of the voltage-current curve when outward currents of high intensity (> 0.1 µA) are passed. The cause of this phenomenon, which seems to be connected with activation processes (cf. JENERICK 1959), will not be dealt with in this work.

The changes in voltage-current relation imply that the effective resistance of the fibre has been reduced. Most likely this reduction can be referred to a change in transverse membrane resistance. However, since it is well known that ethanol passes rapidly into cells (OVERTON 1902) an ethanol effect on the internal resistance has also to be considered. An attempt has been made to estimate this effect as per following, on the basis of the assumption that the ethanol effects on the resistivity of Ringer's solution are comparable to those on the internal resistivity of the muscle cell, in spite of the considerably lower value of the latter (BOZLER and COLE 1935).

A series of determinations, by an ac bridge method, of the resistivity of Ringer's solution showed that ethanol contents of 0.5 M and 1.0 M raised the resistivity values by 10 and 16 per cent respectively. Paying no regard to ionic distribution changes secondary to increased permeability, the influence of such an increase of the internal resistivity on the V/I ratio can be estimated by means of the equation 2 and the membrane constants given in the paper by FATT and KATZ (1951). The calculations showed that an increase of the internal

resistance by 10 or 16 per cent would result in an increase of the effective resistance of the fibre by 5 and 8 per cent respectively However the experimental data actually obtained show a decrease of the ratio V I by about 30 and 50 per cent at the corresponding ethanol content in the muscle baths. It seems therefore, on the basis of the data available at present, that the ethanol effects are mainly to be ascribed to a change in transverse resistance of the muscle fibre membrane.

Discussion

In a brief report on the influence of various toxic substances on the membrane potential of frog muscle fibres measured by means of intracellular technique, TAUC (1952) stated that the effect of 1 M ethanol on the membrane potential was quite insignificant, no depolarization being recorded during the first 15 min and a change of about 10 per cent being observed after 90 min. It has not been possible to compare TAUC's results with those obtained in the present investigation, as the experimental data given in his report are too scarce. It can only be suggested as a possibility that the difference in results is due to differences in the oxygenation of the muscle baths. In the present series of experiments it has been found that ethanol-induced depolarization takes place very slowly under anoxic conditions and is then of the same order of magnitude as reported by TAUC.

The results obtained in earlier studies of demarcation potentials in frog nerve exposed to ethanol (GALLEGO 1948, POSTERNAK and MANGOLD 1949) correspond in some aspects with those found in the present investigation using direct measurements of membrane potential changes in muscle. Thus, recordable changes appear at around the same threshold dose of about 0 1 M, and both in nerve and muscle there is an increase in depolarization with increasing ethanol content. In view of the different recording techniques used, it is however not possible to make quantitative comparisons of the membrane potential changes observed in the two types of tissues.

There is a close correspondence between the ethanol concentration necessary to produce the changes demonstrated in membrane potential and the concentration which, as has been shown by BLUME (1925) results in a lowering of the threshold on direct stimulation of frog muscle. To the threshold rise appearing at higher ethanol content correspond an increased depolarization and a further reduction of the membrane resistance. In analogy with GALLEGO's results on nerve quoted above, both the initial increase and the subsequent reduction of the muscle excitability may be explained by a reduction of the membrane potential.

According to generally accepted views (cf. USSING 1960) the potential difference between the inside and the outside of the muscle fibre is related to the concentration gradients of the diffusable ions and their permeability coefficients. The increase in membrane conductance during ethanol action, implying an

increase of the membrane permeability to one or more ions, may thus be presumed to be a primary cause of the changes of the membrane potential during ethanol exposure. The fact that an increase in the permeability to potassium or chloride ions will tend to increase the membrane potential, whereas an increase in the permeability to sodium ions will cause a depolarization of the membrane, seems to suggest that the membrane permeability to sodium ions is affected during ethanol exposure. However before conclusive evidence can be obtained whether ethanol affects the permeability to any particular ion, it is obviously necessary to undertake experiments especially designed to evaluate the effects of ethanol on the membrane permeability to different ions.

The author is greatly indebted to Dr C. R. Skoglund for continual advice and encouragement and to Mr L. Haapanen for construction of apparatus.

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References

Adrian, R. H., The effect of internal and external potassium concentration on the membrane potential of frog muscle. *J. Physiol.* (Lond.) 1956. *133.* 631—658.

Biedermann, W., Über die durch chemische Veränderung der Nervensubstanz bewirkten Veränderungen der polaren Erregung durch den elektrischen Strom. *S.-B. Akad. Wiss. Wien, math.-nat. Kl.* 1881. *83.* 289—340.

Blume, W., Vergleichende Untersuchungen über die erregbarkeitsteigernde und lähmende Wirkung einiger Narkotika am peripheren Nervenstamm, am Rückenmark und am motorischen Nervenende des Frosches. *Arch. exp. Path. Pharmak.* 1925. *110.* 46—65.

Blumenthal, A., Über die Wirkung verwandter chemischer Stoffe auf den quergestreiften Muskel. *Pflügers Arch. ges. Physiol.* 1896. *62.* 513—542.

Bozler, E. and K. S. Cole, Electric impedance and phase angle of muscle in rigor. *J. cell. comp. Physiol.* 1935. *6.* 229—241.

Fatt, P. and B. Katz, An analysis of the end-plate potential recorded with an intra-cellular electrode. *J. Physiol.* (Lond.) 1951. *115.* 320—370.

Furshpan, E. J. and D. D. Potter, Transmission at giant motor synapses of the crayfish. *J. Physiol.* (Lond.) 1959. *145.* 289—325.

Gallego, A., On the effect of ethyl alcohol upon frog nerve. *J. cell. comp. Physiol.* 1948. *31.* 97—106.

Haapanen, L., A direct coupled amplifier for electrophysiological investigations. *Acta physiol. scand.* 1953. *29.* Suppl. 106. 157—160.

Haapanen, L., G. M. Kolmodin and C. R. Skoglund, Membrane and action potentials of spinal interneurons in the cat. *Acta physiol. scand.* 1958. *43.* 315—348.

Haapanen, L. and D. Ottoson, A frequency compensated unit for recording with microelectrodes. *Acta physiol. scand.* 1954. *32.* 271—280.

Humboldt, F. A. von, Versuche über die gereizte Muskel- und Nervenfaser. Band II. *Decker und Compagnie,* Posen, und *Heinrich August Rottmann,* Berlin 1797.

Hutter, O. F. and S. M. Padsha, Effect of nitrate and other anions on the membrane resistance of frog skeletal muscle. *J. Physiol.* (Lond.) 1959. *146.* 117—132.

Jenerick, H., The control of membrane ionic currents by the membrane potential of muscle. *J. gen. Physiol.* 1959. *42.* 923—930.

KOCHMANN, M., Narcotica der Fettreihe. Handb. exp. Pharmakol. Vol. 2. *Julius Springer* Berlin 1936.

LUCAS, K., The effect of alcohol on the excitation, conduction and recovery processes in nerve. *J. Physiol.* (Lond.) 1913. *46.* 470—505.

OVERTON, E., Beiträge zur allgemeinen Muskel- und Nervenphysiologie. *Pflügers Arch. ges. Physiol.* 1902. *92.* 115—280.

POSTERNAK, J. M. and R. MANGOLD, Action de narcotiques sur la conduction par les fibres nerveuses et sur leur potentiel de membrane. *Helv. physiol. pharmacol. Acta* 1949. *7.* C55—56.

TASAKI, I., E. H. POLLEY and F. ORREGO, Action potentials from individual elements in cat geniculate and striate cortex. *J. Neurophysiol.* 1954. *17.* 454—474.

TAUC, L., Potentiel de repos de la fibre musculaire soumise à l'action de diverses substances toxiques. *C. R. Soc. Biol.* (Paris) 1952. *146.* 1666—1668.

USSING, H. H., The alkali metal ions in isolated systems and tissues. In *Handb. exp. Pharmakol.* vol. 13. The Alkali Metal Ions in Biology. Part I. Springer Verlag, Berlin, Göttingen, Heidelberg 1960.

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Stratification of Absorbed Solutions in Mesenterial Veins[1]

By

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Abstract

ÖSTLING, G. *Stratification of absorbed solutions in mesenterial veins.* Acta physiol. scand. 1961. *52.* 254—266. — Stratification in the mesenterial venous blood of solutions which have been absorbed from the small intestine was demonstrated. Isotonic and hypotonic sodium phosphate solution and water were transported in the upper and hypertonic glucose and saline solutions in the lower streamlines in the mesenterial veins. These observations show that the blood stream is laminar and that the streamline flow demonstrated previously in the portal vein after injection of dyes etc. also occurs in the mesenterial part of the venous portal circulation after intestinal absorption. No stratification could be demonstrated in the mesenterial veins after absorption of isotonic glucose and sodium chloride solution. The reasons for the presence and absence of stratification are discussed and different absorption mechanisms are considered. It is suggested that stratification depends on "intestinal short-cut absorption". According to this hypothesis, under certain conditions translocation from the intestinal lumen of intraintestinally instilled solutions to the capillaries takes place via gaps between the mucosal epithelial cells. These gaps are produced by expulsion of cells from the epithelial border during the absorption.

Stratification in the peripheral circulation of intravascularly administered solutions and suspensions occurs under certain conditions (ÖSTLING 1952). As these observations have been published only in Swedish the phenomena will be described briefly here. Examples are given of flotation, sedimentation and absence of stratification of solutions in the mesenterial vein after their absorption from the small intestine. An attempt is made to explain these phenomena.

[1] A preliminary report of this work was presented at the IX Scandinavian Physiological Congress 1957.

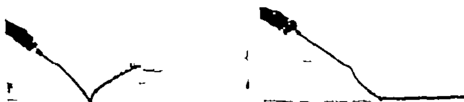


Fig 1 (left) *Flotation*. when Indian ink diluted with w ter is injected slowly into plastic tube with flowing 20 per cent saline solution, the dye rises and is carried in the topmost streamlines.

Fig. 1 (right) *Sedimentation*. when Indian ink diluted with 20 per cent saline solution is injected slowly int plastic tube with flowing water the dye settles and moves in the lowest streamlines.

The stratification phenomenon

Fig 1 illustrates the flotation and sedimentation phenomena. Flotation originates when a solution or suspension flows slowly into a laminar (streamline) stream of fluid of higher specific gravity If on the other hand, the fluid flowing in slowly has a higher specific gravity than the fluid in the plastic tube, sedimentation occurs. If the stream in the tube is not laminar but turbulent, no stratification occurs. It can be seen that the Indian ink introduced is distributed in eddies through all the strata in the plastic tube.

Stratification can also be demonstrated intravascularly in both test animals and man (Östling 1952) If suspensions of Indian ink are injected slowly into a well-exposed and illuminated jugular vein in rabbit, flotation n the blood stream can be seen when the Indian ink is diluted with water and sedimentation when the Indian ink is diluted with 20 per cent saline solution. When thin blood vessels are used for stratification experiments the blood is allowed to run from the vessel some distance from the injection site into a test tube via a needle. When water-diluted Indian ink is used, the topmost streamlines of the blood which run from the needl are black and the black color accumulates in the topmost strata of the blood in the collection tube. When Indian ink of higher specific gravity than blood is used the black color is found in the lowest streamlines and the Indian ink runs down and accumulates on the bottom of the tube. If radioactive phosphate is used instead of Indian ink n the experiment the greatest part of the radioactivity accumulates n the topmost or th lowest strata, depending on the specific gravity of the solutions. Using labelled phosphate solutions, stratification has been established following slow injections into peripheral veins and arteries, and also in the femoral and renal veins after injection into the femoral and renal arteries respectively (Östling 1952) These observations concur with the generally held view that the blood stream in arteries and veins is mostly laminar (Burton 1952) and seem to indicate that streamline flow also occurs in capillaries.

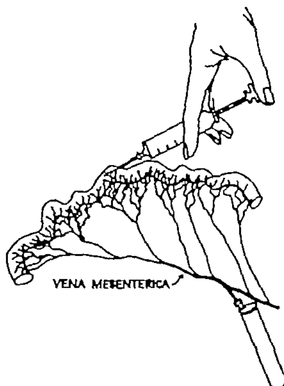


Fig 2. *Sketch illustrating the absorption experiments. A needle is introduced into the mesenterial vein at point where it drains a 10—15 cm long portion of the small intestine. 5 ml of the test solution is then instilled intraluminally into the middle of this section of the intestine and the blood from the vein is collected into test tubes.*

Stratification occurs in the blood stream after slow intravascular instillation of solutions. During absorption from intestine there is also a slow translocation into the blood vessels. The question of whether solutions stratify after absorption from the intestine has not been studied

Methods

Heparinized rabbits weighing 2—2.5 kg were used in the absorption experiments. The animals were anesthetized with an intravenous injection of 8—10 ml of 25 per cent urethane solution or by ether inhalation. The anesthetic used did not affect the experiments. As the urethane injections produced hemolysis, ether anesthesia was employed when examining absorption of pure water

The abdomen was opened and a suitable intestinal loop exposed. The part of the ileum lying about 20 cm orally from the cecum was used most frequently In few experiments the jejunum bout 20 cm from the stomach was employed This part of the intestine was more difficult to use because the mesenterial veins are thinner and more fragile here than in the ileal tract. An injection needle of the same diameter as the vessel was inserted in the mesenterial vein at a point where it drained 10—15 cm long section of the small intestine (Fig. 2) Immediately afterwards 5 ml of the test solution was rapidly injected into the middle of the selected portion of the intestine. The intestine was handled as little as possible throughout the experiment. It was neither rinsed nor emptied in any way The experiments were not affected by the degree of

filling of the intestine or by starvation for periods of different length prior to the experiment. Neither were the exterior temperature of the intestinal loop nor the temperature of the solution injected into it of any significance in the range between room temperature and 38° C.

Immediately after the injection of the test solution into the gut the blood that ran from the needle in the mesenterial vein was collected into the 5 ml test tubes. Depending on how the experiment succeeded, 4—12 tubes full of blood were obtained. In successful experiments the first tubes filled in 10—15 sec. The flow of blood then gradually slowed down and the last tubes were changed after 1—3 min. In experiments where the flow was sluggish the first tubes filled in 1—2 min and last in 5—10 min. It was important that the needle had the same internal diameter as the vein, and that the blood from the needle ran down in an unbroken stream along the inner side of the test tube. After filling, the test tubes were placed in a rack, care being taken not to let shaking disturb possible stratification. Immediately the blood sampling was completed, the uppermost fourth of the sample was withdrawn with pipette. The following fourths were then drawn off in turn. The contents of the test tubes were thus divided into four roughly equal strata. Each separated stratum thus obtained was then mixed by shaking vigorously

The following solutions were employed in the absorption experiments
Hypotonic sodium phosphate solution (0.15 g Na_2HPO_4 $2H_2O$/100 ml H_2O)
Isotonic sodium phosphate solution (1.5 per cent)
Isotonic glucose solution (5 per cent) without and with admixture of phosphate
Hypertonic glucose solution (50 per cent) without and with admixture of phosphate
Isotonic sodium chloride solution (0.9 per cent) with admixture of phosphate
Hypertonic sodium chloride solution (20 per cent) with admixture of phosphat
Distilled water and tap water with and without admixture of carrier-free P^{32}

The hypotonic and isotonic sodium phosphate solutions contained 300—700 μC of radioactive phosphate. The glucose and saline solutions were labelled by adding 0 1—0.5 ml (300—700 μC) of isotonic sodium phosphate solution. The osmolarity of the hypertonic solutions thus varied slightly from one experiment to another but was always hypertonic in relation to the blood plasma. In some of the experiments using water 300—700 μC of P^{32} was added in the form of couple of drops of carrier-free P^{32} solution (obtained from Amersham, England)

The radioactivity in the blood samples was measured with the aid of a mica window Geiger Müller tube. For this purpose 0.02 ml aliquots of whole blood were pipetted onto round discs of filter paper which were glued to the bottom of aluminium planchets. In the experiments with glucose, the glucose content of the layers was determined by the method of HAGEDORN and JENSEN (1954) In the experiments with distilled water and tap water the blood samples were centrifuged at 2,000 rpm. Whether or not the plasma had stained red by hemolysis was assessed by mere inspection.

The stratification of the solutions used in this paper was controlled after slow injection direct into rabbit vein. Flotation in venous blood was observed with distilled and tap water hypotonic sodium phosphate solution and isotonic sodium phosphate, glucose and sodium chlorid solutions sedimentation was established with hypertonic glucose and sodium chloride solutions.

Results

Flotation

Ten rabbits were used in the experiments. Table I shows the stratification in the blood of the mesenterial vein in one of the experiments in which isotonic sodium phosphat solution was injected into the intestinal lumen. The radio-

Table I P^{32} flotation in mesenteric venous blood after injection of isot ic odi m ph ph te solut n into the small intestine

	Number of test tube									
	1	2	3	4	5	6	7	8	9	10
Stratum I (topmost) cpm/0.02 ml	87	124	225	413	902	1,893	3,332	4,626	6,342	6,606
Stratum II cpm/ 0.02 ml.	44	81	83	197	299	897	1,291	2,130	2,858	4,259
Stratum III cpm/ 0.02 ml	21	19	21	52	100	235	369	792	835	1,272
Stratum IV cpm/ 0.02 ml.	8	6	7	32	59	164	356	540	594	678
Sampling time in sec.	20	25	30	35	45	50	60	65	80	[illegible]

Table II P^{32} flotation in mesenteric venous blood after injection of hyp t d m pho phate s lutio into the small intestine

	Number of test tube									
	1	2	3	4	5	6	7	8	9	10
Stratum I cpm/ 0.02 ml	81	332	845	828	862	1 450	2,068	2,446	2,917	1,707
Stratum II cpm/ 0.02 ml	53	193	400	544	591	628	649	970	1,523	1,914
Stratum III cpm 0.02 ml	24	102	210	287	434	339	396	493	613	481
Stratum IV cpm/ 0 02 ml	7	96	151	186	206	244	320	204	345	228
Sampling time in sec.	10	20	20	25	25	35	35	50	70	85

activity is obviously greatest in the topmost stratum of each tube, decreasing towards the bottom. Flotation was observed in all the experiments. In some the flotation was slightly more and in other slightly less pronounced than in the case described in Table I

Eight experiments were made with hypotonic sodium phosphate solution. Flotation was as pronounced as with isotonic phosphate solution. Table II shows the results of one of the experiments.

Twenty nine experiments were made with distilled water or tap water Table III gives the results of an experiment with distilled water mixed with carrier-free P^{32} solution. Flotation of radioactivity and water was observed in this experiment. The red color of the plasma in the uppermost layers derived

Table III Flotation in mesenterial venous blood after injection of distilled water containing carrier-free P^{32} into the small intestine

	Number of test tube						
	1	2	3	4	5	6	7
Stratum I cpm/0.02 ml	+ 300	++ 3,354	++ 4,047	++ 5,304	++ 9,894	+++ 6,045	++++ 12,423
Stratum II, cpm/0.02 ml.	+ 142	+ 1 769	+ 3,042	++ 3,364	++ 4,260	++ 2,646	++ 2,700
Stratum III, cpm/0.02 ml.	29	1 131	2,013	3,039	+ 2,565	+ 1,695	++ 1,063
Stratum IV cpm/0 02 ml.	31	864	1,563	2,316	2,230	+ 1,230	+ 1,377
Sampling time in seconds	20	30	35	40	45	55	60

The + symbols indicate the intensity of red-staining of the plasma.

from hemoglobin liberated by hemolysis. Flotation — indicated by visible red-staining of plasma — was not as regular a phenomenon as in the experiments with isotonic or hypotonic sodium phosphate solution. In 5 exp red staining was not observed in a single one of the blood samples taken. In 15 exp. red flotation was observed in all the tubes and in the remaining 11 tests the topmost layer of plasma stained red in 1—4 of the blood samples. In 3 of these 11 exp the water was labelled with carrier-free P^{32} solution and typical P^{32} flotation was established in each test tube although flotation of color was seen only in 1—1—4 tubes, respectively These findings indicate that the water had risen to the upper strata but the hemolysis was so weak that it was impossible to detect the red color with the naked eye.

Conclusion Isotonic and hypotonic sodium phosphate solutions and water are carried in the upper streamlines of the blood in the mesenterial veins after absorption from the small intestine.

Sedimentation

Fifteen rabbits were given hypertonic glucose solution containing P^{32} Sedimentation was observed in every tube in all the experiments. Table IV gives the result for an experiment in which the glucose content and radioactivity of the layers were determined. Both glucose and radioactivity showed sedimentation. No quantitative correlation was observed between glucose and radioactivity sedimentation in this or the other experiments. In some experiments, in which 50 per cent glucose solution without P^{32} was used the glucose sedimentation was of the same magnitude as in Table IV

Table IV. Sedimentation in mesenterial venous blood after injection of hypertonic glucose solution containing P^{32} into the small intestine

	Number of test tube 1	2	3	4	5
Stratum I, Glucose content: mg/100 ml	158	146	146	162	174
Stratum II, Glucose content: mg/100 ml.	158	178	146	178	226
Stratum III, Glucose content mg/100 ml	172	178	206	232	232
Stratum IV Glucose content mg/100 ml	190	194	208	240	268
Stratum I, P^{32} content cpm/0.02 ml	1	4	9	20	40
Stratum II, P^{32} content: cpm/0.02 ml	8	20	40	81	131
Stratum III P^{32} content cpm/0.02 ml.	30	43	90	160	266
Stratum IV P^{32} content cpm/0.02 ml	69	94	133	218	463
Sampling time in seconds	40	45	60	93	150

Table V. P^{32}-sedimentation in mesenterial venous blood after injection of hypertonic sodium chloride solution containing P^{32} into the small intestine

	Number of test tube 1	2	3	4	5	6	7
Stratum I, cpm/0.02 ml.	43	54	62	58	71	100	187
Stratum II cpm/0.02 ml	52	68	98	99	180	256	[illegible]
Stratum III cpm/0.02 ml	45	83	137	264	425	429	657
Stratum IV cpm/0.02 ml	58	129	265	613	681	727	1,031
Sampling time in seconds	30	43	63	83	100	120	160

Table V is from an experiment with hypertonic saline solution with P^{32} addition. The radioactivity was highest at the bottom of the columns of blood. The series comprised 7 exp. Sedimentation was observed in all of them. In 2, however, there were individual tubes in which the radioactivity was almost evenly distributed throughout the column of blood.

The radioactivity only and not the NaCl content was determined in these experiments with hypertonic saline solution containing P^{32}. The results obtained from the experiments with glucose solution and water containing P^{32} lend support to the assumption that the sedimentation demonstrated did not comprise solely the labelled phosphate but the solution as a whole.

Conclusion. Hypertonic glucose and saline solution are transported in the lower streamlines of the blood in the mesenterial veins after absorption from the small intestine.

Table VI. V P^{32}-stratification in mesenteral venous blood after injection of i t l gl c l ti containing P^{32} into the small intestine

	Number of test tube 1	2	3	4	5	6	7	8
Stratum I cpm/0.02 ml.	215	240	454	429	634	675	662	571
Stratum II, cpm/0.02 ml	278	458	444	534	567	633	693	683
Stratum III, cpm/0.02 ml.	254	581	496	557	563	645	818	742
Stratum IV cpm/0.02 ml.	183	296	571	517	645	620	754	762
Sampling time in seconds	25	30	30	35	40	50	55	65

Table VII. V P^{32}-stratification in mesenterial venous blood after injection of i t i di m kl id l ti containing P^{32} into the small intestine

	Number of test tube 1	2	3	4	5	6	7
Stratum I cpm/0.02 ml.	618	473	386	398	428	390	438
Stratum II, cpm/0.02 ml.	516	509	419	434	420	398	435
Stratum III, cpm/0.02 ml	435	556	79	518	454	436	403
Stratum IV cpm/0.02 ml.	316	744	668	542	465	389	320
Sampling time in seconds	25	30	35	30	40	45	50

Absence of stratification

Isotonic glucose solution with P^{32} added was used in 11 exp. Table VI which gives the results for one of them, gives no evidence of the occurrence of stratification. Nine of the experiments gave similar results irrespectively of whether radioactivity or glucose content was determined. Sedimentation appeared to occur in 2 of the tests. It was not so pronounced, however as when hypertonic glucose and saline solution was administered. As mentioned previously isotonic glucose solution rises to the upper streamlines after direct slow injection into the peripheral veins.

Table VII shows the result of an experiment with isotonic sodium chloride solution containing P^{32}. No stratification of radioactivity could be established. Of the 12 exp. performed, 11 gave the same result. One experiment showed an equally pronounced flotation as with isotonic or hypotonic phosphate solution. The result was obtained too late to permit an examination of the injection solution used to eliminate the possibility that the solution had been wrongly prepared.

Conclusion. Isotonic glucose and sodium chloride solutions showed no stratification and were evenly distributed in the mesenterial venous blood stream after absorption from the small intestine.

Discussion

The present investigation shows that isotonic and hypotonic sodium phosphate solutions of lower specific gravity than blood and water are transported in the upper streamlines of the mesenterial venous blood stream after absorption from the intestine. Hypertonic glucose and saline solutions of higher specific gravity than blood are carried in the lower streamlines after translocation from the intestinal lumen. The laminar (streamline) blood flow in the portal vein, which prevents blood from the different internal organs from mixing prior to arrival in the liver (COPHER and DICK 1928, HAHN *et al.* 1943) thus also occurs in the mesenterial veins during absorption.

The intravascular hemolysis observed by LEE (1954) after absorption of water is verified by the present observation of water flotation, which causes hemolysis in the topmost streamlines in the mesenterial veins. Flotation of water in blood *in vitro* has previously been demonstrated (ÖSTLIND and NYBERG 1952)

After translocation from the intestine to the veins certain solutions are carried further in the upper or lower streamlines of the mesenterial blood. Thus, the solutions are distributed in the blood stream just as if they had entered the circulation in a slow flow from the intestinal lumen via narrow openings (pores) The fluid circuit theory of INGRAHAM and VISSCHER (1938) assumes that there is a flow of fluid through pores of different sizes between the intestinal lumen and the capillaries and vice versa. The idea of the existence of pores in the intestinal mucosa has been revived recently (CURRAN and SOLOMON 1957 PARSONS and WINGATE 1958 DURBIN *et al* 1958) CURRAN and SOLOMON (1957) write as follows "The epithelial membrane may be described in physical terms as equivalent to a membrane with uniform negatively charged right circular pores of 36 Å radius, occupying 0 001 per cent of the surface area." This size of the radius is of interest because PAPPENHEIMER (1955) calculated that for membranes with a greater effective radius of pore than c. 20 Å the net flow of water through diffusion is negligible compared with hydrodynamic flow caused by hydrostatic or osmotic pressure. DURBIN *et al* (1958) suggest that the pores in the ileum are intercellular Earlier physiological investigations thus indicate that there may be pores in the intestinal mucosa through which the intestinal contents flow into the capillaries.

The ultrastructure of the intestinal mucosa and intestinal capillaries has been studied in the last few years by electron microscopy (ZETTERQVIST 1956 BENNETT *et al.* 1959 PALAY and KARLIN 1959 a) No pores forming a direct connection between the intestinal lumen and the capillaries have been established. Fenestrations or pores of a radius of 100—250 Å occur in the endothelial cells of the capillaries. Counting from the lumen, between the intestinal lumen and these pores there are (1) the apical surface and striated

border of the epithelium, (2) the cytoplasm of the epithelium, (3) the basal or lateral surfaces of the epithelial cells, (4) the basement membrane under lying the epithelium, (5) the loose connective tissue of the lamina propria (6) the basement membrane surrounding the capillaries" (PALAY and KARLIN 1959 a) For flow of intestinal fluid from the lumen into the capillaries to be possible it is necessary to postulate that these tissues can be passaged in some way so that the unchanged intestinal contents enter the pores of the capillary endothelium.

A possible way in which unchanged intestinal contents can pass via the epithelial layer is pinocytosis (PALAY and KARLIN 1959 b, CLARK 1959 CRANE 1960) As there is no confirmation that pinocytosis plays a role in the absorption of solutions employed in the present study this transport mechanism must be rejected for the present.

Another way in which the intestinal contents might circumvent the epithelial cells can be deduced from RITTER's (1957) investigation of expulsion of epithelial cells from intestinal mucosa. Microscopy of living intestinal mucosa at 50—100 × magnifications showed that epithelial cells were expelled when different nutritive solutions were dropped on the mucosa. When 1—2 cells were freed small gaps were produced and were closed almost instantaneously by filling with adjacent cells. In the event of major defects in the epithelium (4—5 cells) it took ½—1 hour before the openings were covered. According to RITTER, a continuous expulsion and regeneration of epithelial cells occurs in the villi during absorption. Observations by the isotope technique that the epithelial cells of the intestin regenerate in 24—48 hours concur with this finding (LEBLOND *et al.* 1957 HOOPER *et al* 1958)

The possibility that absorption can occur via a short-cut through gaps left by expelled epithelial cells is supported by some observations. JANKOWSKY (1925) found numerous epithelial cells in the Ringer's solution used to rinse the small intestine of rabbit. He estimated that 5 000—7,000 cells were freed per minut and sq cm. Expulsion of epithelial cells thus occurs in rabbits, the species employed in the present investigation. RITTER (1959) observed that the extent of expulsion varied with the substances administered. Hyper tonic solutions such as 50 per cent glucose and 10 per cent saline cause considerably more active expulsion of cells than the same solutions in isotonic concentrations. With hypertonic solutions the defects in the epithelial layer in villi increase in number and size and repair more slowly This gives the intestinal contents greater chances of coming into contact with the subepithelial tissues. Hypertonic glucose and saline solutions showed pronounced stratification in the present experiments after absorption, whereas no stratification could be demonstrated when the corresponding isotonic solutions were used. One may therefore *assume a correlation* between vigorous cellular expulsion and stratification, on the one hand, and limited cellular expulsion and no stratification on the other hand. It can be inferred from this

that solutions which provoke pronounced expulsion of cells can pass the epithelial border of the intestinal wall and flow via the openings produced.

But there is still the translocation through the basement membranes and the interjacent loose connective tissue before the pores of the capillaries are reached. The basement membranes, judging by electron microscopy seem to be almost homogenous but of low opacity and to have neither pores nor meshwork. It has been pointed out recently however that the basement membranes are composed of a condensation of fine fibrills (PALAY and KARLIN 1959 a). It seems that the basement membranes have a very subtle structure which is either difficult to demonstrate by existing techniques or which is destroyed postmortally. The possibility that fluid may flow through the basement membranes by an unknown mechanism (electro-osmosis?) cannot be disregarded entirely especially as it has not been possible to demonstrate any opaque structural barrier. The hypothetical short-cut absorption via gaps in the epithelial border of the villi produces flotation or sedimentation depending on whether the specific gravity of the intestinal contents is lower or higher than that of the capillary blood. Solutions which are carried evenly dispersed in the venous stream following the absorption pass, according to this hypothesis, all the layers of mucosa including the epithelial cells which obstruct direct flow from the intestinal lumen to the capillaries. Short-cut absorption would thus seem to involve stratification, whereas translocation through the epithelial cells inhibits it.

Stratification can theoretically originate also in each junction of the mesenterial veins and of the veins and the capillaries when two blood streams of different specific gravity combine. The stem of the vein from which the blood samples were taken drained a 10—15 cm long piece of the intestine. The test solutions were always injected into the middle of the intestinal sector (see fig. 3). No peristalsis was observed in at least 90 per cent of the experiments. The 5 ml injected could therefore not be distributed evenly throughout the lumen, and the concentration was greatest around the injection site. The veins of this part consequently were able to absorb more of the instilled solution than the veins of the outer parts of the intestinal loop employed. Hence although the conditions for stratification should always have been present it could not be demonstrated with either isotonic glucose or saline solution. The possibility that stratification originates in the greater branches of the venous tree close to the stem used for the sampling must therefore be precluded. For the same reasons, the origin of stratification at all other levels in the mesenterial veins and capillaries can also be eliminated.

The stratification of solutions in mesenterial venous blood following absorption from the intestine is difficult to account for by current absorption theories. Short-cut absorption, i.e. passage of the intestinal contents via gaps left by expelled cells in the marginal epithelial layer of the mucosa, offers a hypothetic explanation of the phenomenon. Stratification has been demonstrated in the large mesenterial veins, but it probably occurs in the entire mesenterial venous system. If this is true, it must be assumed that there is a

flow of the intestinal fluid into the capillaries via the pores in the capillary endothelium. The powers that drive the stream cannot be envisaged. Hydrostatic pressure can probably be ruled out as peristalsis was seen very seldom during the present absorption experiments and as stratification was usually more pronounced the later the blood samples were taken (see the tables).

Absorption via the intercellular area between adjacent epithelial cells has been discussed previously. Nothing has been observed to confirm such a route of absorption (ZETTERQVIST 1956). Short-cut absorption via gaps in the epithelial coating of the villi has not been proposed earlier to the present author's knowledge. RITTER (1957), who saw gaps in the living mucosa, was unable to establish any correspondence in the histological preparations obtained postmortally although he examined hundreds of sections. In the classical histological picture the villi are covered by an unbroken row of epithelial cells. This does not, however, preclude the appearance of openings at rare in the epithelial border during absorption. The villi are made up of soft, mobile tissue which performs pumping movements. It is therefore possible that the villi collapse after death, pressing the epithelial cells together and effacing the gaps.

These attempts to account for the origin of the stratification phenomenon are based on weak evidence. The hypothesis of intestinal short-cut absorption must therefore be regarded as merely a working hypothesis.

I am greatly indebted to miss KERSTIN FROSTELL for technical assistance and docent RALPH GRÄSBECK for criticism of the manuscript.

Addendum

C. P. LEBLOND (Classical technics for the study of the kinetics of cellular proliferation in *The kinetics of cellular proliferation*, edited by F. STOHLMAN, GRUNE & STRATTON, New York and London 1959, p. 33—37. See also Anat. Rec. 1958. *132*. p. 247) has observed in man, cat, rabbit and mouse at the tips of the villi *extrusion zones* with numerous irregularities, interrupted striated border and shriveled cells pushed above the regular epithelial lining. The occurrence of such extrusion zones seems to support the intestinal short cut absorption hypothesis.

References

BENNETT, H. S., J. H. LUFT and J. C. HAMPTON, Morphological classifications of vertebrate blood capillaries. *Amer. J. Physiol.* 1959. *196*. 381—390.

BURTON, A. C., Laws of physics and flow in blood vessels. *Ciba Found. Symp.* J. & A. Churchill Ltd. London 1952. p. 70.

CLARK, S. L. The ingestion of proteins and colloidal material by columnar absorptive cells of the small intestine in suckling rats and mice. *J. biophys. biochem. Cytol.* 1959. *5*. 41—50.

COPHER, G. H. and B. M. DICK, "Stream line" phenomena in the portal vein and the selective distribution of portal blood in the liver. *Arch. Surg.* 1928. *17*. 408—419.

CRANE, R. K., Intestinal absorption of sugars. *Physiol. Rev.* 1960. *40*. p. 816.

Curran, P. F. and A. K. Solomon, Ion and water fluxes in the ileum of rats. *J. gen. Physiol.* 1957. *41.* 143—168.

Durbin, R. P., P. F. Curran and A. K. Solomon, Ion and water transport in stomach and intestine. *Advanc. biol. med. Phys.* 1958. *6.* 1—36.

Hagedorn, H. C. and N. Jensen, In *Hawk's Practical Physiological Chemistry.* McGraw-Hill Book Company. New York 1954. p. 577.

Hahn, P. F., W. D. Donald and R. C. Grier, The physiological bilaterality of the portal circulation. Stream line flow of blood into the liver as shown by radioactive phosphorus. *Amer. J. Physiol.* 1945. *143.* 105—107.

Hughes, W. L., V. P. Bond, G. Brecher, E. P. Cronkite, R. B. Painter, H. Quastler and F. G. Sherman, Cellular proliferation in the mouse as revealed by autoradiography with triated thymidine. *Proc. nat. Acad. Sci.* (Wash.) 1958. *44.* 476—483.

Ingraham, R. C. and M. B. Visscher, Further studies on intestinal absorption with the performance of osmotic work. *Amer. J. Physiol.* 1938. *121.* 771—785.

Jankowsky, M. A., Über die Emigration auf den Schleimhäuten des Verdauungskanals. *Frankfurt. Z. Path.* 1925. *32.* 238—244.

Leblond, C. P., N. B. Everett and B. Simmons, Sites of protein synthesis as shown by radioautography after administration of S^{35}-labelled methionine. *Amer. J. Anat.* 1957. *101.* 225—271.

Lee, J. S., Intravascular hemolysis during water absorption from small intestine. *Amer. J. Physiol.* 1954. *178.* 103—110.

Östling, G. Flotation i blod. *Nord. Med.* 1952. *47.* 886—887.

Östling, G., Simultaneous intestinal absorption of solute and solvent. *Acta physiol. scand.* 1957. *42.* Suppl. 111—112.

Östling, G. and W. Nyberg, Dye flotation in blood. *Scand. J. clin. Lab. Invest.* 1952. *4.* 201—203.

Palay, S. L. and L. J. Karlin, An electron microscopic study of the intestinal villus. I. The fasting animal. *J. biophys. biochem. Cytol.* 1959 a. *5.* 363—371.

Palay, S. L. and L. J. Karlin, An electron microscopic study of the intestinal villus. II. The pathw[illegible]y of fat [illegible]bsorption. *J. biophys. biochem. Cytol.* 1959 b. *5.* 373—383.

Pappenheimer, J. R., Passage of molecules through capillary walls. *Physiol. Rev.* 1953. *33.* 387—423.

Parsons, D. S. and D. L. Wingate, Fluid movements across wall of rat small intestine in vitro. *Biochim. biophys. Acta* 1958. *30.* 666—667.

Ritter, U. Histophysiologische Untersuchungen am Darmepithel während der Verdauung. *Gastroenterologia* (Basel) 1957. *88.* 153—171.

Ritter, U. Personal communication. 1959.

Zetterqvist, H., The ultrastructural organization of the columnar absorbing cells of the mouse jejunum. *AB Godvil.* Stockholm 1956.

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Human Serum Cholinesterase as a Sialo-Protein

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Abstract

Svensmark, O. *Human serum cholinesterase as a sialo-protein.* Acta physiol. scand. 1961. 52. 267—275. — Incubation of human serum with sialidase (neuraminidase, receptor destroying enzyme) results in a marked decrease of the mobility of cholinesterase without affecting the enzymatic activity as indicated by the splitting of acetylcholine and butyrylcholine. The results suggest that human serum cholinesterase is an acid glycoprotein containing several sialic acid residues per molecule. In normal serum sialidase could not be detected whereas spinal fluid showed weak sialidase activity. In a single pool of serum from patients a marked sialidase activity was observed.

Knowledge of the chemical properties of human serum cholinesterase (pseudocholinesterase, unspecific cholinesterase) is scarce as this enzyme has not yet been isolated in a pure state. In a purified preparation considerable amounts of carbohydrate were found but the impurities still present made it impossible to identify the cholinesterase as a glycoprotein (Surgenor and Ellis 1954). Even from impure preparations information concerning the properties of cholinesterase can be obtained when its enzymatic activity is used to trace the protein. Thus, the electrophoretic mobility determined on paper at pH 9 was between that of α_2- and of β-globulins (Grégoire and Derrien 1952). In chromatography of serum on diethylaminoethyl-cellulose (DEAE-cellulose) cholinesterase appeared between albumin and the acid glycoprotein coeruloplasmin (Svensmark 1961) suggesting that the enzyme is an acid protein.

The acidity of acid α_1-glycoprotein is due to sialic acid residues which can be split off the protein by bacterial sialidase (neuraminidase) whereby the mobility of the protein is considerably reduced (Popenoe and Drew 1957).

I have investigated whether the acidity of cholinesterase is due to sialic acid by using the effect of sialidase on the electrophoretic mobility of the protein as a criterion for the presence of sialic acid residues. This was feasible because the enzymatic activity of cholinesterase was not abolished by the treatment with sialidase.

Materials and Methods

Normal human serum was used as a source of cholinesterase. *Cholinesterase activity* was determined at 37° C by electrometric titration at pH 7.4 in a medium consisting of 87 mM NaCl and 4.3 mM $MgCl_2$ with acetylcholine iodide and butyrylcholine bromide (4.3 mM) as substrates.

Determination of migration distance of cholinesterase in paper electrophoresis. An LKB electrophoresis equipment (LKB Fabriksaktiebolag, Stockholm) was used. The paper strips (Schleicher & Schüll no. 2043 bmgl) were wetted with the buffer (barbital pH 8.6, $\mu = 0.075$) and blotted on filter paper. To obtain comparable values, the conditions of electrophoresis were kept identical from experiment to experiment. The paper was equilibrated with the power (3.5 V/cm) switched on for 3 hours ($\pm$ 10 min) before the samples were applied, and the time of electrophoresis was 18 hours ($\pm$ 10 min). The spots of serum placed on the paper were small (1 μl) to reduce the error in determining the migration distance (distance from the point of application to the center of the spot). Electrophoresis was performed at room temperature.

Visualization of cholinesterase. The wet strips were immersed in a solution of 2 M NaCl, 50 mM NaH_2PO_4 adjusted to pH 7.3 with 1 N NaOH, 10 mM $MgCl_2$, 0.2 mg/ml α-naphthyl acetate and 0.2 mg/ml Fast Blue B Salt (diazonium salt of o-dianisidine, G. T. Gurr Ltd., London). In the course of 2—10 minutes at room temperature a red spot appeared on the paper at the site of the cholinesterase. The paper strips were finally rinsed in distilled water and dried (Porter 1957).

Electrophoresis of serum proteins. On the strips to be used for evaluation of the distribution of protein 20 μl of serum were applied as a narrow transversal band. After electrophoresis the strips were dried at 110° C to denature the protein and stained for 10 min in a saturated solution of Amidoschwarz 10 B in a mixture of 45 per cent (v/v) methanol, 45 per cent (v/v) water and 10 per cent (v/v) acetic acid. Then the strips were washed in several changes of methanol-water-acetic acid. The electropherogram was recorded on a paper strip scanner with logarithmic output.

Sialidase. The sialidase preparation used was Receptor Destroying Enzyme (lot no. 23, Behringwerke, Germany) which according to the manufacturer is dried filtrate from cultures of Vibrion cholerae. Two ml of human serum dialyzed against distilled water and one ml of 50 mM NaH_2PO_4 adjusted to pH 6.8 with 1 N NaOH were incubated with 1—25 mg per ml serum of the sialidase preparation at 25° C for variable lengths of time. A drop of toluene was added to prevent bacterial contamination. A sample of buffered serum kept for the same length of time but without sialidase served as control.

Chromatography of serum. Serum was chromatographed on diethylaminoethyl-cellulose (DEAE-cellulose, Whatman DE 50). One ml of serum dialyzed against 5 mM NaH_2PO_4 adjusted to pH 7.0 with 1 N NaOH was applied to a column (10 × 120 mm) and eluted with a decreasing pH and increasing salt gradient from 5 mM phosphate buffer to 0.5 M NaCl and 50 mM NaH_2PO_4 (Sober et al. 1956). The eluate was collected in 5 ml fractions; protein was determined by measuring the transmittance at 280 mμ (Beckman spectrophotometer model DU) and the cholinesterase activity was assayed by the semi-quantitative procedure described above: 10 μl spots were applied to filter paper and the

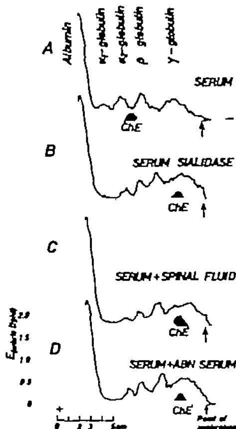


Fig 1 *The effect of sialidase on the mobility of cholinesterase* Paper electrophoresis at pH 8 6 of (A) serum, (B) serum + sialidase (10 mg/ml), (C) serum + spinal fluid concentrated 100 times by ultrafiltration (50 µl/ml) and (D) serum + abnormal serum (20 µl/ml) The incubation time was 10 days 25 C. One paper strip was stained for protein and another for cholinesterase. Black areas indicate the localization of cholinesterase activity (ChE).

paper immersed in the α-naphthyl acetate "Fast Blue B Salt" solution. The intensity of the red color indicated the cholinesterase activity By this method 100 samples could be assayed in less than half an hour As control of the pplied pH and salt gradient the conductivity and pH of the effluent were determined (Conductivity Meter CDM 2 and pH Meter PHM 22, Radiometer Copenhagen)

Results

Effect of sialidase on cholinesterase

1 *Change f mobility* After incubation at pH 6.8 with sialidase the mobility of cholinesterase decreased with time until the mobility corresponded to that of the slowest part of the γ-globulin. The mobilities of α and β-globulins were reduced as well, although not to the same extent as the mobility of cholinesterase (Fig 1 A and B) About one-twentieth of the protein was precipitated during incubation.

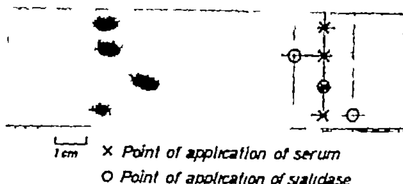


Fig. 2. Interaction between sialidase (0.1 mg per spot) and cholinesterase (2 µl serum per spot) during paper electrophoresis at pH 8.6. The strip was stained for cholinesterase. The mobility of cholinesterase was reduced only when serum and sialidase were applied together

The ratio of the migration distance of the sialidase-treated cholinesterase (at minimal mobility) to the migration distance of untreated cholinesterase was 0.35 ± 0.005 (11 exp.)

2. *Rate of mobility change.* To investigate the rate of change of the mobility produced by sialidase the migration distance of cholinesterase was determined by electrophoresis at pH 8.6 after various times of incubation. The period of incubation to be considered was the time from incubation to termination of the electrophoretic run as the reaction between sialidase and cholinesterase continued during electrophoresis. That sialidase and cholinesterase were not separated during electrophoresis was indicated by the following experiment

Four spots of serum were applied along a transversal line on the paper strip and three spots of sialidase (0.1 mg in 2 µl) were placed in front of, superposed on and behind the serum spots (Fig 2) Electrophoresis was performed for 18 hours at pH 8 6 and the strips were stained for cholinesterase. The mobility of cholinesterase was reduced when sialidase and serum were applied together This indicates that the reaction between sialidase and cholinesterase continues during electrophoresis. Sialidase placed in front of or behind the serum spot did not interact with cholinesterase. Thus, the mobilities of sialidase and of cholinesterase were roughly identical and the reaction between the enzymes can proceed during at least part of the electrophoresis.

The ratio of migration distances of sialidase-treated to untreated cholinesterase was determined at various time intervals after incubation with 1—25 mg/ml of the sialidase preparation (Fig 3) With a given sialidase concentration the mobility of cholinesterase decreased gradually with time. The rate of the mobility change increased with increasing concentration of sialidase and minimal mobility was only reached after more than six days of incubation.

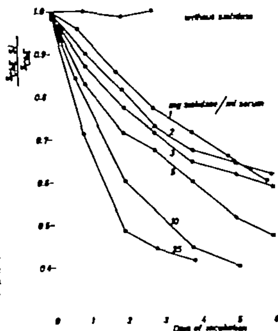


Fig. 3. Influence of sialidase on the mobility of cholinesterase as a function of incubation time. The ratio of migration distance of sialidase-treated and untreated cholinesterase ($S_{ChE\ s.}/S_{ChE}$) was used as measure of the mobility. Human sera were incubated at 25° C with 1—25 mg/ml sialidase at pH 6.8 and the migration distance determined by paper electrophoresis pH 8.6. The curve for 5 mg/ml is averaged from 4 experiments and those for 10 and 25 mg/ml from 2 experiments.

3. *Effect of sialidase on the DEAE-cellulose chromatogram of cholinesterase.* The decrease in acidity of cholinesterase after treatment with sialidase was also evident from a comparison of the DEAE-cellulose chromatograms of sialidase treated and untreated human sera (Fig. 4). The sialidase-treated cholinesterase was eluted together with neutral protein (γ-globulin) while the untreated cholinesterase appeared after albumin.

4. *Effect of sialidase on the enzymatic activity of cholinesterase.* The appearance of a red color in the visualization procedure for cholinesterase (see p. 268) indicates that the enzyme can still split α-naphthyl acetate after treatment with sialidase. A quantitative expression of the enzymatic activity of cholinesterase was obtained by electrometric titration with acetylcholine and butyrylcholine as substrates. The cholinesterase activity remained unchanged after treatment with sialidase (Table 1).

Occurrence of sialidase in human serum and spinal fluid

Human serum normally did not exhibit detectable sialidase activity. This was indicated by the identical mobility of cholinesterase in 50 serum samples immediately after withdrawal and after keeping at room temperature for up to two months.

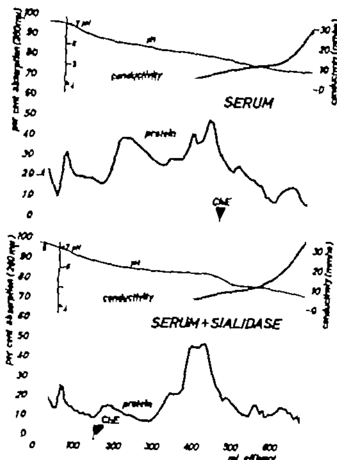


Fig. 4 Chromatography of treated and sialidase-treated human serum on DEAE-cellulose columns. Serum was incubated at 25 C with 10 mg/ml sialidase for 10 days. The heights of the black columns indicate the activities of cholinesterase (ChE) as determined by the semiquantitative spot-assay. The upper curves indicate the pH and conductivity of the effluent.

Spinal fluid on the other hand, changed the mobility of human serum cholinesterase and of α and β-globulins in the same way as the sialidase preparation (Fig 1 C). This effect was found in spinal fluid from three different pools with an average protein content of 0.38 mg/ml and normal electropherograms. In seven experiments the minimal ratio of migration distance of spinal fluid-treated and untreated cholinesterase was 0 34 ± 0 007 i e. the same as obtained with the sialidase preparation. The activity as estimated from the effect on the mobility of cholinesterase was low. One ml of spinal fluid corresponded to 0 1 mg of the sialidase preparation.

In a single serum pool from hospitalized patients a reduced mobility of cholinesterase was observed. The mobilities of α- and β-globulin were also decreased. The ratio of the migration distance of cholinesterase from this pool to that of cholinesterase from all other sera investigated was 0 34 i e. the same order as found for sialidase-treated cholinesterase. Addition of small amounts

Table I. Absence of an effect of sialidase preparation (10 mg/ml serum) on the enzymatic activity of human serum cholinesterase. Cholinesterase activity was determined titrimetrically at pH 7.4 and 37° C

Serum	Cholinesterase activity [µmoles/(min. ml)]				Days of incubation at 25° C and pH 6.8	$S_{ChE\,sa}$ / S_{ChE}
	Acetylcholine		Butyrylcholine			
	Untreated serum	Sialidase-treated serum	Untreated serum	Sialidase-treated serum		
1	3.10	2.94	6.24	6.[illegible]6	8	0.34
1	3.10	3.12	6.36	6.30	60	0.34
2	3.82	3.88	7.88	7.85	8	0.36
2	3.97	3.85	7.92	8.00	9	0.38
3	3.93	3.92	8.36	8.23	11	0.36

duplicate or triplicate determinations.

The ratio of migration distances of sialidase-treated and untreated cholinesterase as determined by paper electrophoresis at pH 8.6.

of the abnormal serum to normal serum (e. g. 1 µl/ml) produced similar reduction in the mobilities of cholinesterase and of α- and β-globulin as obtained with sialidase. The ratio of migration distances of cholinesterase after and before incubation with the abnormal serum was 0.34 ± 0.003 (20 exp.). The electropherogram of serum incubated with the abnormal serum was the same as after incubation with sialidase (Fig. 1 D). The effect of the abnormal serum was abolished by heating to 90° C for 5 min whereas dialysis had no influence on the effect. These findings indicate the presence of sialidase in the abnormal serum sample. The effect of 1 µl of the abnormal serum on the mobility of cholinesterase corresponded approximately to the effect of 1 mg of the sialidase preparation. Unfortunately it has not been possible to find the donor of this serum.

Discussion

The findings reported in this study suggest that the acidity of human serum cholinesterase is due to the presence of sialic acid bound to the protein in such a way that its release by sialidase does not expose charged sites. Although the number of sialic acid residues could not be estimated, the gradual decrease of mobility of cholinesterase during incubation with sialidase which proceeds without distinct steps, indicates that several molecules of sialic acid are involved. The isoelectric point of normal cholinesterase was displaced by treatment with sialidase from less than 3 to about 7 (SVENSMARK and KRISTENSEN, unpublished). A similar decrease in mobility has been described for acid α₁-glycoprotein after

treatment with sialidase (POPENOE and DREW 1957) Fifteen to sixteen molecules of sialic acid per molecule of this protein are released with a simultaneous increase of the isoelectric point from 2 7 to 5 0 Similar effects of sialidase on other serum sialoproteins (transferrin, orosomucoid, haptoglobin and coeruloplasmin) have been observed (ONGLEY EYLAR and SCHMID 1958, LAURELL and BRÖNNESTAM 1959) and the effect on the α- and β-globulins of serum has been described by SCHULTZE and SCHWICK (1957) and by LAURELL (1959)

It is remarkable that the enzymatic activity of cholinesterase is retained after removal of acid residues from the molecule, i. e. under the conditions investigated a substantial change in charge did not affect the enzymatic activity These findings are in contrast to the inactivation of "Follicle Stimulating Hormone and human Menopausal Gonadotropin by enzymic release of N-acetyl-neuraminic acid and glycolyl-neuraminic acid respectively (GOTTSCHALK, WHITTEN and GRAHAM, 1960 GOT and BOURRILLON 1961)

That sialidase activity could not normally be detected in human serum is in agreement with the observation by Warren and Spearing (1960) They found, however a weak sialidase activity in commercial bovine and human plasma fractions (α- and β-globulins) Provided that our three pools of spinal fluid were not contaminated by abnormal spinal fluid, sialidase is a normal constituent of spinal fluid. This is also suggested by the occurrence of free sialic acid in spinal fluid (UZMAN and RUMLEY 1956) Similarly the occurrence of a cholinesterase fraction with a mobility comparable to that of γ-globulin in spinal fluid (SVENSMARK 1958, 1960 1961) indicates the presence of sialidase.

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References

GOT R. and R. BOURRILLON Enzymic release of sialic acid and inactivation of human menopausal gonadotropin. *Nature* (Lond.) 1961 *189*, 234—235

GOTTSCHALK, A., W K. WHITTEN and E. R. B. GRAHAM, Inactivation of follicle-stimulating hormone by enzymic release of sialic acid. *Biochim. biophys. Acta* 1960 *38*, 183—184

GRÉGOIRE, J and Y DERRIEN, Répartition de l'activité cholinestérasique dans les fractions *protéiques des sérums d'homme et de cheval séparées par électrophorèse sur papier C. R. Soc. Biol.* (Paris) 1952. *146*, 1491—1493

LAURELL, A.-B Neuraminidase-like factors in cultures of pneumococci, alpha-streptococci and pasteurella pseudotuberculosis. *Acta path. microbiol. scand.* 1959 *47* 182—190.

LAURELL, A.-B. and R. BRÖNNESTAM, Influence of alpha-streptococci, and pasteurella pseudotuberculosis on human serum glycoproteins. *Acta path. microbiol. scand* 1959. *47* 429—434.

ONGLEY J. L., E. H. EYLAR and K. SCHMID, Action of pneumococcus neuraminidase on acid glycoprotein. *Vox Sang New Series* 1958. 3. 50—51

PORTH, L., Esterase activity of serum protein fractions. *Hung Acta physiol.* 1957 *11* 39—44.

POPENOE, E. A. and R. M DREW The action of an enzyme of clostridium perfringens on orosomucoid. *J biol. Chem.* 1957 *228*, 673—683.

SCHULTZE, H. E. and G. SCHWICK, Immunochemischer Nachweis von Proteinveränderungen unter besonderer Berücksichtigung fermentativer Einwirkung auf Glyko- und Lipoproteide. *Behringwerk Mitteilungen.* 1957 33, 11—38.

SOBER, H. A., F. J. GUTTER, M. M. WYCKOFF and E. A. PETERSON, Chromatography of proteins II. Fractionation of serum protein on anion-exchange cellulose. *J. Amer. chem. Soc.* 1956. *78.* 756—763.

SURGENOR, D. M. and D. ELLIS, Preparation and properties of serum and plasma proteins. Plasma cholinesterase. *J. Amer. chem. Soc.* 1954 *76.* 6049—6051

SVENSMARK, O., Localisation of cholinesterases in the protein fractions of cerebrospinal fluid. *Abstr. IV. Int. Congr. Biochem.*, Wien, 1958, p. 171

SVENSMARK, O., Undersøgelser over tre cholinesterasefraktioner i spinalvædske. *Nord. Med.* 1960. *64* 1023.

SVENSMARK, O., Cholinesterases in human spinal fluid and brain. *Acta physiol. scand.* 1961 *52.* 372—378.

UZMAN, L. L. and M. K. RUMLEY, Neuraminic acid as a constituent of human cerebrospinal fluid. *Proc. Soc. exp. Biol.* (N. Y.) 1956. *93.* 497—501

WARREN, L. and C. W. SPEARING, Mammalian Sialidase (Neuraminidase). *Biochem. biophys. res. Comm.* 1960. *3.* 489—492.

Acta physiol. scand. 1961. 52. 276—290

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Urinary Excretion Patterns for Substances with Simultaneous Secretion and Reabsorption by Active Transport

By

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Abstract

BERGLUND, F. *Urinary excretion patterns for substances with simultaneous secretion and reabsorption by active transport.* Acta physiol. scand. 1961. *52.* 276—290. — Substances, which are actively secreted and reabsorbed by the renal tubules, show different excretion patterns. Except for potassium, they all follow a "secretion proximal" pattern, i. e. secretion takes place at a site proximal to reabsorption or through identical cells. Thiosulfate, creatinine and uric acid are typical examples. — For *thiosulfate* secretion Tm = reabsorption Tm. At low plasma levels its clearance is low, at higher levels the clearance equals glomerular filtration rate. — For *creatinine* secretion Tm > reabsorption Tm, its clearance is low at low plasma levels, but exceeds glomerular filtration rate at high levels. The dependence of "endogenous creatinine clearance" on glomerular filtration rate and renal blood flow is discussed. — A similar pattern is shown by *uric acid* in the rabbit. In man, uric acid reabsorption Tm > secretion Tm, the net result being reabsorption at all plasma levels.

The *formation of urine comprises three main processes, namely* 1. ultrafiltration of plasma in the glomeruli, 2. tubular reabsorption of solutes and water, 3. tubular secretion of solutes. Reabsorption and secretion by the tubules are effected by "active transport" and/or diffusion.

Active transport by the renal tubules is usually supposed to occur in one direction only, *i. e.* either as reabsorption or secretion. There are, *however,* substances which are both reabsorbed and secreted in certain species, *e. g.* thiosulfate, creatinine, uric acid and potassium. On the basis of experiments

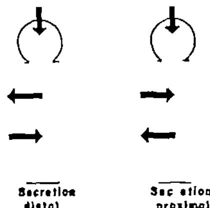


Fig. 1 Relative localization of secretion and reabsorption in the renal tubules.
"[illegible]": solute is reabsorbed proximally, and secreted at more distal site.
"[illegible]": solute is secreted proximally, and reabsorbed at more distal site, or at the same site as secretion.
"Distal" and "proximal" do not signify localization to distal or proximal tubules.

with thiosulfate, BERGLUND, HELANDER and HOWE (1960) have formulated a secretion proximal" and secretion distal" concept. The proofs relating to secretion proximal" are partly new but most originate from papers published during the last two decades, though not fully interpreted at the time. Often the proof has been of pharmacological nature inhibition of secretion has revealed the existence of simultaneous secretion and reabsorption (as for thiosulfate, creatinine and uric acid) The following presentation shows that secretion proximal often gives rise to characteristic excretion or clearance patterns.

Relative sites of secretion and reabsorption

Depending on the sites of secretion and reabsorption, relative to each other a secretion distal and a secretion proximal" pattern may be distinguished (Fig 1) *Secretion distal*" involves filtration in the glomeruli, reabsorption at a proximal site in the tubules, and finally secretion at a more distal site. Everything secreted appears in the urine.

A substance following the *secretion proximal*" pattern is filtered in the glomeruli and secreted at a proximal site in the tubules. Reabsorption takes place at a more distal site. Both filtered and secreted substance may thus be reabsorbed. — If a substance is secreted and reabsorbed at identical sites in the tubules, any amount secreted will immediately be available for reabsorption. This alternative is therefore included in secretion proximal"

The terms secretion distal" and secretion proximal" should not be interpreted in terms of localization to the distal or proximal tubules they only signify the site of secretion relative to reabsorption.

Relative magnitudes of transfer maxima (Tm s)

The shape of the excretion and clearance curves depends on the relative magnitudes of reabsorption Tm and secretion Tm. The first condition to be

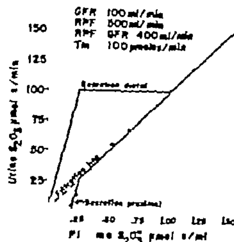


Fig. 2. Urinary excretion of thiosulfate in female dog, with GFR around 90 ml/min. Each ● is average of three clearance periods, adjusted to GFR 100 ml/min. — Theoretical lines show expected urine thiosulfate for "filtration", "secretion distal" and "secretion proximal". For assumptions and calculations, see Table I. Modified from BERGLUND, HELANDER & HOWE (1960).

discussed is that of equal Tm s in both directions (section I) This section illustrates the difference between secretion distal" and "secretion proximal" Sections II and III describe cases with unequal Tm s for secretion and reabsorption, but only for substances with secretion proximal" Potassium is therefore not included.

In the calculations which follow it has been assumed that both secretion and reabsorption show typical Tm curves, i. e the substance is completely extracted from peritubular blood or from tubular urine until Tm is reached.

I Reabsorption Tm = Secretion Tm. Example Thiosulfate in man and female dog

In man (BUCHT 1949) and dog (FOULKS *et al.* 1952) thiosulfate is both secreted and reabsorbed. In the female dog the Tm in each direction is around 125 µmoles/100 ml glomerular filtrate (BERGLUND *et al.* 1960) Fig 2 shows expected urinary excretion of thiosulfate assuming filtration only or secretion distal" or secretion proximal" The figure is based on the assumptions and calculations in Table I

In the *secretion distal"* pattern filtered thiosulfate would be completely reabsorbed at low plasma levels. Secreted thiosulfate would appear in the urine and soon reach a Tm. With further increasing plasma levels, filtration and reabsorption of thiosulfate increase together When reabsorption Tm is reached, filtered thiosulfate would appear in the urine, and the excretion would then follow the filtration line"

If thiosulf [illegible] ollowed the [illegible] *ion proximal"* pattern, both filtered and secreted thi [illegible] would be r [illegible] rbed at low plasma levels. When reabsorption Tm [illegible] , the ex [illegible] would rise steeply because of addition both from g [illegible] filtration [illegible] ular secretion. When secretion Tm is reached, [illegible] woul [illegible] the filtration line"

Table I Expected urinary excretion of thiosulfate at selected plasma levels

Modified from BERGLUND, HELANDER and HOWE (1960)

Assume: Reabsorption Tm = Secretion Tm = 100 μmoles/min.
GFR = 100 ml/min.
RPF = 500 ml/min.
RPF — GFR = 500 — 100 = 400 ml/min.
Then Filtered S_2O_3 = 100 × Plasma S_2O_3
Secreted S_2O_3 = 400 Plasma S_2O_3
"Secretion distal" Urine S_2O_3 = (Filtered S_2O_3 — Reabsorbed S_2O_3) + Secreted S_2O_3.
"Secretion proximal" Urine S_2O_3 = (Filtered S_2O_3 + Secreted S_2O_3) — Reabsorbed S_2O_3.

Plasma thiosulfate	Urine thiosulfate	
	"Secretion distal"	"Secretion proximal"
μmoles/ml	μmoles/min.	μmoles/min.
0.15	(15 — 15) + 60 = 60	(15 + 60) — 75 = 0
0.20	(20 — 20) + 80 = 80	(20 + 80) — 100 = 0
0.25	(25 — 25) + 100 = 100	(25 + 100) — 100 = 25
1.00	(100 — 100) + 100 = 100	(100 + 100) — 100 = 100
1.25	(125 — 100) + 100 = 125	(125 + 100) — 100 = 125

It is of interest to compare "secretion distal" and "secretion proximal". Secretion becomes saturated at the same plasma level (0.25 μmoles/ml) in both cases. Reabsorption becomes saturated at high plasma levels in "secretion distal" (only filtered load is reabsorbed) but at low plasma levels in "secretion proximal" (filtered + secreted load is reabsorbed). The "filtration line" is intercepted at different plasma levels, namely when both secretion and reabsorption become saturated.

Urinary excretion data in the dog (Fig. 2) show that thiosulfate follows the "secretion proximal" pattern (BERGLUND *et al.* 1960). Data from competition studies further indicate that secretion and reabsorption are effected by identical cells.

II. "Secretion proximal" Secretion Tm > Reabsorption Tm. Examples: Creatinine in man, rat, goat. Uric acid in rabbit. PAH and diodrast in Necturus.

This alternative shows a characteristic excretion pattern. At low plasma levels the clearance is low, sometimes below glomerular filtration rate (GFR). As the plasma level increases, the clearance first rises above and then again approaches GFR.

Creatinine follows such a pattern in the goat (Fig. 3, reconstructed from LADD *et al.* 1957). At a plasma level of 1 mg % its clearance equals inulin clearance. At 3 mg % the clearance is twice as high, and then gradually decreases. Probenecid, p-(dipropylsulfamyl)-benzoic acid, partly inhibits the secretion. —

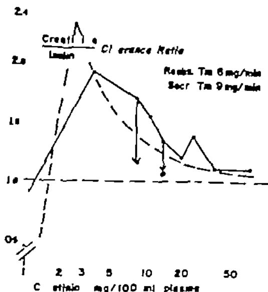


Fig 3. ●—●—● creatinine clearance in the goat ○ ○ after probenecid. Reconstructed from LADD et al. (1957). — — — theoretical clearance curve, cf. Table II

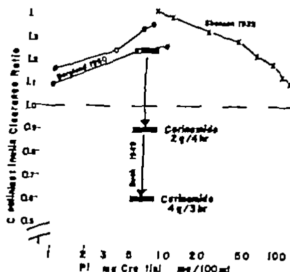


Fig. 4 Creatinine clearance in man. Maximum creatinine/inulin clearance ratio (1.3—1.4) around plasma level of 8—10 mg %. Carinamide® depresses clearance ratio below unity — Constructed from data of SHANNON (1935), BUCHT (1949) and two own experiments (Berglund 1960**). — Technical data on Berglund 1960** Two identical twins. Each (represents three 10-minute periods. Water diuresis 10—14 ml/min. Inulin clearance 118 and 119 ml/min respectively Creatinine analysis according to LAUSON (1954) inulin according to HEYROVSKY (1956)

Table II shows expected clearances of creatinine, for GFR = 100 ml/min, reabsorption Tm = 6 mg/min and secretion Tm = 9 mg/min. The Tm values were chosen by trial and error The theoretical curve shows fair agreement with the experimental data (Fig 3)

In man the clearance follows a flatter curve than in the goat (Fig 4) The maximum creatinine/inulin clearance ratio is only 1 4 and is reached at a plasma level as high as 8—10 mg %. On the other hand it is possible to block

Table II Expected urinary excretion and clearance of creatinine at selected plasma levels

Assume: Reabsorption Tm = 6 mg/min.
Secretion Tm = 9 mg/min.
GFR = 100 ml/min.
RPF = 500 ml/min.
RPF — GFR = 500 — 100 = 400 ml/min.
Urine creatinine: (Filtered + Secreted) — Reabsorbed.

Plasma creatinine mg/100 ml	Urine creatinine mg/min.	Clearance ml/min.
1.2	1.2 + 4.8 — 6 = 0	0
1.5	1.5 + 6 — 6 = 1.5	100
2.25	2.25 + 9 — 6 = 5.25	233
3	3 + 9 — 6 = 6	200
5	5 + 9 — 6 = 8	160
10	10 + 9 — 6 = 13	150
Varying glomerular filtration rate		
Assume: GFR = 50 ml/min. RPF = 500 ml/min. RPF — GFR = 450 ml/min.		
1.5	0.75 + 6.75 — 6 = 1.5	100
Assume: GFR = 200 ml/min. RPF = 500 ml/min. RPF — GFR = 300 ml/min.		
1.5	3.0 + 4.5 — 6 = 1.5	100
Varying renal plasma flow		
Assume: GFR = 100 ml/min. RPF = 450 ml/min. RPF — GFR = 350 ml/min.		
1.5	1.5 + 5.25 — 6 = 0.75	50
Assume. GFR = 100 ml/min. RPF = 450 ml/min. RPF — GFR = 450 ml/min.		
1.5	1.5 + 6.75 — 6 = 2.25	150

the secretion so completely (or selectively?) with carinamide[a] p-(benzylsulfonamido)benzoic acid, that reabsorption dominates.

In the white rat and in the kangaroo rat similar conditions prevail (Table III) Endogenous creatinine clearance"[b] is practically identical with the inulin clearance at elevated levels of creatinine, its clearance is almost twice as high. Probenecid depresses the creatinine/inulin clearance ratio towards unity

Dependence of creatinine clearance on glomerular filtration rate and renal blood flow. Endogenous creatinine clearance"[b] is often used as an approximate measure of GFR, on the assumption that creatinine at normal plasma levels is filtered only As it, however is also secreted and reabsorbed its clearance may not always equal GFR. Theoretically the excretion and clearance of endogenous

Table III Creatinine/inulin clearance ratios at various plasma levels of creatinine in unanesthetized rats and kangaroo rats

Species	Plasma Creatinine mg/100 ml	Creatinine/Inulin clearance ratio	Investigator
Rat	[1]0.29 ± 0.01	1.10	PETERS (1959)
	22.3 ± 1.0	1.98 ± 0.22	
	(Means ± standard error)		
Rat	20 → 150	1.90 → 1.35	FOOT. (1952)
	Highest ratios at low plasma levels. Ratios depressed towards 1.0 by probenecid		
Kangaroo rat (*Dipodomys merriami*)	[1]0.3	1.1 (?)	SCHMIDT NIELSEN (1954)
	1.6	1.0	
	4	1.3	
	11	1.7	
	31	1.4	

[1] "Endogenous creatinine"

creatinine" should actually be independent of GFR, because any increase of the amount filtered will be counterbalanced by a reduction of the creatinine available for tubular secretion, and *vice versa* (Table II) This is illustrated by the two cases in Table IV in which GFR was increased by oxyphyllin®

Table IV Effect of oxyphyllin® on inulin and PAH clearances, and on the excretion of endogenous creatinine. From tables 16 and 17 in EK *(1955)*

	Clearance Inulin ml/min.	Clearance PAH ml/min.	Urine creatinine mg/min.
	Normal A5		
Control	166	801	1.11
	158	834	1.09
	186	969	1.58
Oxyphyllin 1.35 g i. v	357	1 002	1.47
	347	911	1.27
	Hypertension Hyp 14		
Control	141	462	1.22
	130	599	1.33
	130	591	1.25
Oxyphyllin 1.35 g i.	309	431	1.51
	266	384	1.33

Table V Effect of nepresolin® on clearances in the goat

Dose of nepresolin	Plasma creatinine mg/100 ml	Clearance PAH ml/min.	Inulin ml/min.	Creatinine ml/min.	Ratio Creatinine/Inulin Clearance
Exp. 6					
0	0.99	232	48	56	1.15
25 mg L	0.91	410	63	79	1.26
25 mg L	1.06	456	54	66	1.19
Exp. 7					
0	1.07	336	68	71	1.05
25 mg L	0.99	559	58	72	1.29

Female goats, about 40 kg body weight. The figures are average of 3, 3 and 2 clearance periods in exp. 6; 3 and 5 periods in exp. 7

(theophyllin-1-aminopropanol) (Ek 1955) After injection of oxyphyllin the inulin clearance doubled, *PAH clearance remained unchanged, whereas* the excretion of endogenous creatinine" increased by less than 20 % in fair agreement with the calculations in Table II.

In contrast, the endogenous creatinine clearance" should be highly sensitive to changes in renal blood flow so that a 10 % change in renal plasma flow (RPF) should change the endogenous creatinine clearance by 50 % (Table II) To test the sensitivity to changes in RPF experiments were done with nepresolin® (Ciba 1 4-dihydrazinophtalazine methane sulfonate) in goats (Table V) In two experiments PAH clearance doubled, whereas inulin clearance increased or decreased slightly the creatinine/inulin clearance ratio increased in both experiments, but much less than expected. This might be due to less complete extraction of creatinine from the peritubular capillaries, when renal blood flow increased above normal values.

Thus, endogenous creatinine clearance" does not rise significantly when GFR *or* RPF is increased. A marked rise occurs, however when GFR and RPF increase together (Bocht *et al* 1956) This would be expected, because filtered creatinine then increases without reducing the amount available for secretion by the tubules.

When GFR and RPF fall below normal, as in renal insufficiency the endogenous creatinine"/inulin clearance ratio is usually elevated and may reach values as high as 2.2 (Mattas *et al.* 1952) this might partly be due to an increased plasma level, but selective impairment of the reabsorption mechanism cannot be excluded.

When GFR is strongly depressed by circulatory disturbances, as occurs in man in diabetic coma or in morphine-scopolamine intoxication, the exogenous

Table VI Creatinine/Inulin clearance ratios with falling plasma levels of creatinine

Time	Plasma creatinine	Clearances		Ratio Creatinine/Inulin Clearance
		Inulin	Creatinine	
min.	mg %	ml/min.	ml/min.	
		EXP 14		
0	Prime: Inulin 4 g, creatinine 6 g. Infusion, inulin 0 08 g/min, creatinine 0.06 g/min, Na_2SO 0.09 g/min. in H_2O 1,3 ml/min.			
31—60	36	96	106	1.14
137—156	39	*97*	*112*	*1.16*
157	Creatinine discontinued			
280—309	12	88	117	1.33
		EXP 15		
	4 liters H O at — 30 min. and at 168 min.			
0	*Prime and Infusion = Exp. 14, without Na_2SO*			
30—51	42	82	87	1.07
140—159	45	73	88	1.20
160	Creatinine discontinued			
285—310	15	84	116	1.39

Female goat, 40 kg, 3 weeks after pregnancy In exp. 14 osmotic diuresis (1.8—3.8 ml/min.). In exp. 15 water diuresis (9—13 ml/min.), with hemolysis and hemoglobinuria during last hour. Each figure is average of 2—3 periods, of 7—10 minutes duration.

creatinine clearance" falls below the inulin clearance (McCance and Widdowson 1939) This has been ascribed to increased tubular permeability permitting backdiffusion of creatinine. Tubular secretion may however still persist. Thus, in the goat restriction of blood flow to one kidney decreased the creatinine/inulin clearance ratio from 1.5 to 0 75 Probenecid depressed this clearance ratio still further (to < 0.3) evidently by blocking secretion (Ladd *et al.* 1957)

Creatinine clearance with falling plasma levels. When the plasma level of creatinine is allowed to fall from high levels, the creatinine/inulin clearance ratio does not always rise as expected (Shannon 1935 Shannon and Ranges 1941 Ladd *et al.* 1957) In the goat, Ladd *et al.* obtained a ratio of 1 11 at a plasma level of 35 mg %, and 3 hours later a ratio of 0 64 at a plasma level of 12 mg %, reabsorption thus dominating over secretion To further elucidate this, experiments were done in a female goat (Table VI) The plasma level was kept well above 30 mg % for 2 hours and then allowed to fall. The clearance ratio then rose above 1 3 instead of falling below 1 0. This was true both with osmotic diuresis and water diuresis. — The experiments of Shannon and Ranges (1941) were of a different character They showed, in man, that a second dose of creatinine elevated the depressed clearance ratio. Unfortunately

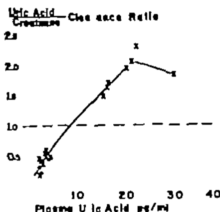


Fig. 5. Uric acid clearance in the rabbit (reconstructed from POULSEN 1955) × × without probenecid, ○ ○ after probenecid 50—150 mg/kg body weight intravenously

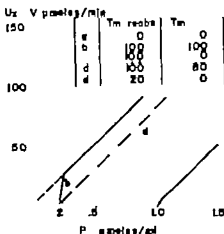


Fig. 6 Urinary excretion of substance X, with secretion proximal", and reabsorption Tm > secretion Tm. GFR = 100 ml/min, RPF = 500 ml/min. Ux V = urinary excretion. Note two possible interpretations of line d.

their plasma levels before the second dose were as low as 4—9 mg %, and after injection 14—21 mg %, so a higher clearance ratio was to be expected in most experiments (*cf* Fig 4) — It is concluded that depression of creatinine/inulin clearance ratios with falling plasma levels is not reproducible and is of doubtful significance.

Uric acid. In the rabbit, the clearance of uric acid follows a similar pattern. At low plasma levels, the uric acid/creatinine clearance ratio is well below 1.0 (Fig 5) At higher plasma levels this ratio reaches a value of 2.3, but is depressed below unity by probenecid (POULSEN 1955)

PAH and Diodrast. In Necturus p-aminohippurate (PAH) and diodrast show net reabsorption at low plasma levels. At higher levels their clearances approach or exceed creatinine clearance (KEMPTER 1959) The pattern fits with secretion proximal" — Autoradiographic studies in Necturus with dio-

drast I[19] show that the cells of the proximal tubules are the exclusive site of intracellular accumulation, and they appear to be the major site of diodrast transport, irrespective of the direction (KINTER, LEAPE and COHEN 1960)

III "Secretion proximal" Reabsorption Tm > Secretion Tm. Example uric acid in man.

This alternative is analyzed in Fig 6. Lines *a* and *be* are identical with "filtration line" and "secretion proximal" in Fig. 2. Line *c* (parallel to *a*) shows expected urinary excretion when reabsorption Tm = 100 µmoles/min and secretion Tm = 0 The reabsorption Tm would be reached at a plasma level of 1 µmole/ml.

For comparison, return to curve *be* reabsorption Tm = secretion Tm = 100 µmoles/min. If the secretion Tm is gradually diminished, line *e* will move to the right and soon originate directly from the abscissa (line *d*) this occurs *at a plasma level of 0.2 µmoles/ml At this point* secretion Tm (now 80 µmoles/min) and reabsorption Tm (still 100 µmoles/min) will be reached simultaneously and line *b* disappears. An identical pattern (line *d*) would be obtained if there were reabsorption alone with a Tm = 20 µmoles/min. A typical reabsorption Tm pattern therefore does not exclude tubular secretion of considerable magnitude.

This may be exemplified by uric acid, which in man is reabsorbed by the tubules. Uric acid appears in the urine before the "Tm" is reached and at normal plasma levels about 10 % of filtered uric acid (filtered + secreted uric acid) escapes reabsorption. YÜ and GUTMAN (1955) reported that uricosuric drugs (sodium salicylate, phenylbutazone, probenecid) in therapeutic doses raise, but in smaller doses lower the excretion of uric acid. They suggested that salicylates etc. increase the net reabsorption of uric acid by inhibiting tubular secretion, and that uric acid, like potassium, is reabsorbed proximally and secreted distally The excretion pattern described above suggests, however a "secretion proximal" pattern similar to that in the rabbit, but with reabsorption dominating at all plasma levels.

Comparative physiology

Simultaneous secretion and reabsorption following the "secretion proximal" pattern has been demonstrated for thiosulfate, uric acid creatinine, PAH and diodrast. Both secretion and reabsorption are probably effected by the proximal tubules, as with diodrast in Necturus. The two-way transport may originate from secretory systems occurring among the vertebrates, complete reversal yielding reabsorption, partial reversal yielding secretion + reabsorption.

This may be exemplified by the transport system for *sulfate* and *thiosulfate* (Table VII) (BERGLUND and FORSTER 1958, BERGLUND *et al* 1960 BUCHT 1949) Both ions are secreted by the aglomerular kidneys of the Lophius. In the dog and in man, sulfate is reabsorbed while thiosulfate is both secreted

Table VII Comparative physiology of substances which follow a "secretion proximal" pattern

Substances	Species	*Secretion.* (+) Inhibitors	*Reabsorption.* (+) Inhibitors
Thiosulfate Sulfate	Goosefish (*Lophius americanus*)	+ Mutual inhibition Carinamide	
	Dog Man	*Thiosulfate only* + Sulfate Carinamide	*Both ions* + Mutual inhibition
Diodrast PAH	Rabbit Man	+ Mutual inhibition	
	Salamander (*Necturus maculosus*)	+ Mutual inhibition	+ Mutual inhibition
Creatinine	Marine fish Bullfrog Chicken Anthropoid apes	+ Phlorizin	
	Rat Goat Man	++ Phlorizin	+
Uric acid	Lizard (*Iguana iguana Shaw*)	+	
	Chicken	+ Probenecid Salicylates	
	Rabbit	++ Probenecid Salicylates	+
	Dog, non-dalmatian		+ Probenecid
	Dog, Dalmatian	++ Probenecid	+ (?)
	Man	+ Probenecid Salicylates	++ Probenecid Salicylates

+ means occurrence of active transport (secretion or reabsorption). ++ signifies the higher Tm in case of bidirectional transport. With secretion ++ and reabsorption + reabsorption may still predominate at low plasma levels (cf. Fig. 5). Absence of + means that transport has not been demonstrated.

and reabsorbed. Still, the two ions seem to be transported by the same carrier They inhibit each other's transport competitively both in the direction of reabsorption and secretion. Carinamide® but not probenecid, inhibits the secretion of both ions.

Similar reciprocal inhibition occurs between *diodrast* and *PAH* (Table VII) *They have been shown to inhibit each other's secretion in rabbit and man* (JOSEPHSON *et al.* 1953 b and a) In Necturus they are both secreted and reabsorbed and mutually inhibit each other's transport in both directions (KINTER 1959)

Creatinine is secreted by the renal tubules in frog (SWANSON 1956) marine fish, chicken and anthropoid apes (for ref. see SMITH 1951 p. 143) It is both secreted and reabsorbed in rat, goat and man, and in these species secretion is inhibited by carinamide® (BUCHT 1949) or probenecid (FINOL 1952 LADD *et al.* 1957) These inhibitors have not been tested in fish, chicken or frog, but most likely the secretion is effected by the same mechanism in all vertebrates involved. Phlorizin depresses the creatinine/inulin clearance ratio in the dogfish, frog, chicken, goat, chimpanzee and man (for ref. see SMITH 1951 pp. 98—99)

Uric acid. Uric acid is largely eliminated by tubular secretion in reptiles and birds, (for ref. see Smith 1951 p. 144)

Various excretion patterns exist in mammals. *Uric acid is both secreted and* reabsorbed in man, but the secretion is masked by the high reabsorption Tm. — In the rabbit reabsorption dominates at endogenous plasma levels (0.2—0.5 mg %) but secretion dominates at elevated plasma levels (Fig 5) — In the dog uric acid is reabsorbed by the tubules. It is, however secreted in the Dalmatian coach hound, in which the clearance may be twice as high as GFR (WOLFSON COHN and SHORE 1950) Mixed breeds (Dalmatian — nondalmatian) may like pure bred dalmatians, be "high uric acid producers" TRIMBLE and KEELER (1938) *noted that* Animals bearing white hairs diffused in their spots have been consistently 'low producers' of uric acid, while those animals which have had 'high uric acid production have had uniform black pigment in their spots" Dalmatian hybrids studied by ONSLOW (1923) showed a similar trend. It may therefore be that "the recessive gene, which converts diffuse ticking or flecking to polka-dotting is the same gene which is responsible for the high uric acid excretion" (ALAN MITCHELL, personal communication 1956)

Secretion of uric acid is in general inhibited by probenecid and salicylates. Reabsorption, when present, may also be inhibited by these drugs (BEYER *et al.* 1951 POULSEN 1955 YÜ and GUTMAN 1955 NECHAY and NECHAY 1959, BERGER, YÜ and GUTMAN 1960) When both secretion and reabsorption are inhibited in man uricosuria results.

The goat experiments were made possible by the helpful collaboration of Doctor BENGT ANDERSSON at Veterinärhögskolan, Stockholm. Doctor LARS OLAS performed one of the twin clearance experiments. Skilled technical assistance was given by Miss MONA HENRIKSSON.

References

BERGER, L., T F YÜ and A. B. GUTMAN, Effect of drugs that alter uric acid excretion in man on uric acid clearance in the chicken. *Amer J Physiol.* 1960 *198*. 575—580

BERGLUND, F and R. P FORSTER, Renal tubular transport of inorganic divalent ions by the aglomerular marine teleost, Lophius americanus. *J gen. Physiol.* 1958. *41* 429—440.

BERGLUND, F C.-G HELANDER and R. B. HOWE, Inorganic sulfate and thiosulfate transport and competition in the renal tubules of the dog. *Amer J Physiol.* 1960. *198*. 586—594.

BEYER, K. H., H. F RUSSO, E. K. TILLSON, A. K. MILLER, W F VERWEY and S. R. GASS, 'Benemid' p-(di-n-propylsulfamyl)-benzoic acid its renal affinity and its elimination. *Amer J Physiol.* 1951 *166*. 625—640

BOJESEN H., On the tubular excretion of thiosulfate and creatinine under the influence of caronamide. *Scand. J clin. Lab. Invest.* 1949 *1* 270—276.

BOJESEN H., J EK, B. JOSEPHSON, B. THOMASSON, E. VARNAUSKAS and L. WERKÖ, Rapid infusion and renal function. *Clin. Sci.* 1956. *15*. 617—633.

EK, J The influence of heavy hydration on the renal function in normal and hypertensive man. *Scand. J clin. Lab Invest.* 1955 *7* Suppl. 19.

FINGL, E., Tubular excretion of creatinine in the rat. *Amer J Physiol.* 1952 *169*. 357—362.

FOULKS, J P BRAZEAU, F. S. KOELLE and A. GILMAN Renal secretion of thiosulfate in the dog. *Amer J Physiol.* 1952. *168*. 77—85.

HEYROVSKY A., A new method for the determination of inulin in plasma and urine. *Clin. chim. Acta.* 1956. *1* 470—474

JOSEPHSON, B., J EK, H. BOJESEN L. WERKÖ, J KALLAS and A. GRIEG, The competition between p-amino-hippuric acid (PAH) and diodrast for renal excretion and extraction in man. *J clin. Invest.* 1953 a. *32*. 1184—1191

JOSEPHSON, B A. GRIEG, G. KAKOMAKOS and J KALLAS, Renal tubular excretion from high plasma levels of para-amino-hippurate (PAH) and diodrast (D) in unanaesthetized rabbits. *Acta physiol. scand.* 1953 b. *30* 11—21

KINTER, W B., Renal tubular transport of diodrast I[131] and PAH in Necturus evidence for simultaneous reabsorption and secretion. *Amer J Physiol.* 1959 *196*. 1141—1149

KINTER, W B., L. L. LEAPE and J J COHEN, Autoradiographic study of diodrast I[131] transport in Necturus kidney *Amer J Physiol.* 1960 *199* 931—941

LADD, M., L. LIDDLE, J A. GAGNON and R. W CLARKE, Glomerular and tubular functions in sheep and goats. *J appl. Physiol.* 1957 *10*. 249—255.

LEME, F On the determination of creatinine in plasma by the Jaffé reaction after adsorption to Lloyd' reagent. *Scand. J clin. Lab. Invest.* 1954 *6*. 325—334.

MATTAR, G H. L. BARNETT, H. MCNAMARA and H. D. LAUSON, Measurement of glomerular filtration rate in children with kidney disease. *J clin. Invest* 1952. *31* 938—946.

MCCANCE, R. A. and E. M. WIDDOWSON, Functional disorganization of the kidney in disease. *J Physiol.* (Lond.) 1939. *95*. 36—44.

NECHAY B. R. and L. NECHAY Effects of probenecid, sodium salicylate, 2.4-dinitrophenol and pyrazinamide on renal secretion of uric acid in chickens. *J Pharmacol.* 1959. *126*. 291—295.

ONSLOW H., Uric acid and allantoin excretion among offspring of Dalmatian hybrids. *Biochem. J* 1923. *17* 564—568.

PETERS, G., Glomeruläre Clearancen, p-Aminohippursäure-Clearance und Diurese bei verschiedenen Diureseformen der nicht narkotisierten Ratte. *Arch. exp. Path. Pharmak.* 1959 *235* 113—142.

POULSEN, H., Inhibition of uric acid excretion in rabbits given probenecid or salicylic acid. *Acta pharmacol.* (Kbh.) 1955. *11* 277—286.

SCHMIDT NIELSEN, B., Excretion of endogenous and exogenous creatinine and of mannitol in the kangaroo rat. *Amer J Physiol.* 1954. *178*. 177—181

SHANNON, J A., The renal excretion of creatinine in man. *J clin. Invest.* 1935. *14* 403—410.

SHANNON, J. A. and H. A. RANGES, On the renal tubular excretion of creatinine in normal man. *J. clin. Invest.* 1941. *20.* 169—176.
SMITH, H. W., The kidney. Structure and function in health and disease. *Oxford Univ. Press*, New York 1951.
SWANSON, R. E., Creatinine secretion by the frog renal tubule. *Amer. J. Physiol.* 1956. *184.* 527—534.
TRIMBLE, H. C. and C. E. KEELER, The inheritance of "high uric acid excretion" in dogs. *J. Hered.* 1938. *29.* 280—289.
WOLFSON, W. Q., C. COHN and C. SHORE, The renal mechanism for urate excretion in the Dalmatian coach-hound. *J. exp. Med.* 1950. *92.* 121—128.
YÜ, T. F. and A. B. GUTMAN, Paradoxical retention of uric acid by uricosuric drugs in low dosage. *Proc. Soc. exp. Biol.* (N. Y.) 1955. *90.* 542—547.

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Augmented Cardiac Contraction, Heart Acceleration and Skeletal Muscle Vasodilatation Produced by Hypothalamic Stimulation in Cats

By

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Abstract

Rosén, A. *Augmented cardiac contraction, heart acceleration and skeletal muscle vasodilatation produced by hypothalamic stimulation in cats.* Acta physiol. scand. 1961 52. 291—308. — Hypothalamic stimulation in cats was found to produce an increase of the contractile force of the right ventricle as measured by a strain gauge arch. Concomitant tachycardia was generally observed. The responses were mediated both by cardiac sympathetic innervation and by catechols released from the adrenals. In some experiments the nervous mechanism dominated, in others the humoral factor. The hypothalamic area, the stimulation of which produced cardiac augmentation, appeared to correspond with "the sympathetic vasodilator area". Muscle vasodilator as well as cardiac augmentative responses appeared concurrently.

The sympathetic vasodilator nerves to the skeletal muscles in dogs and cats can be activated by hypothalamic stimulation. The vasodilatation produced during such stimulation is due to activation of cholinergic fibres and can be markedly reduced or abolished by atropinization of the animal. During central stimulation of the sympathetic vasodilator outflow vasomotor responses are not confined to the blood vessels in the muscles. Vasoconstriction occurs concomitantly in cutaneous and splanchnic regions (Eliasson *et al.* 1951 Lindgren and Uvnäs 1953 Lindgren 1955) together with an increased adrenal release of catechols, mainly adrenaline (Grant *et al.* 1958 Lindgren Rosén and Uvnäs 1959 a). Though the amounts of adrenaline released in most experiments are so minute as to cause only minor vascular effects, if any they may nevertheless suffice to produce metabolic effects (Grant *et al.* 1958, Lindgren, Rosén and Uvnäs 1959 b).

The physiologic significance of the observed pattern of responses to intracerebral stimulation is not known. ELIASSON *et al.* (1951) and UVNÄS (1954) have suggested that activation of the sympathetic vasodilator outflow might occur in fear anger and other situations associated with emotional stress. Recent observations by ABRAHAMS and HILTON (1958) ABRAHAMS, HILTON and ZBRÓZYNA (1960 a and b) and BLAIR *et al.* (1959) have led to similar conclusions. Among other suggestions put forward is the possibility that the vasodilator system is in some way concerned with the initial adjustment of the muscular blood flow to exercise (UVNÄS 1954) The observed increase of muscle blood flow associated with posthaemorrhagic syncope led BARCROFT *et al.* (1944) and BARCROFT and EDHOLM (1945) to suggest that a sympathetically-mediated active vasodilatation occurred.

The aim of this paper is to contribute to elucidation of the functional significance of the sympathetic vasodilator system. It is sought to ascertain whether augmentation of cardiac functions such as contractile force and heart rate accompanies the characteristic peripheral responses to central stimulation of the vasodilator outflow An affirmative finding would tend to support the above mentioned hypothesis of UVNÄS concerning the functional significance of the sympathetic vasodilator system.

The literature contains few data on the influence on cardiac contractile force following hypothalamic stimulation. RUSHMER, SMITH and FRANKLIN (1959) and SMITH *et al* (1960) localized in the canine hypothalamus a few small areas the stimulation of which caused changes in left ventricular performance identical with the responses to treadmill exercise. RUSHMER and collaborators consider that these observations lend weight to the old idea of a central nervous mechanism which causes an increase of cardiac work during the initial phase of muscle activity

Many investigators have observed cardiac acceleration following hypothalamic stimulation, but few data on heart rate responses to hypothalamic stimulation of the sympathetic vasodilator pathway are found in the literature. In a few experiments in which the heart rate was studied tachycardia was observed to accompany the muscle vasodilator response elicited by hypothalamic stimulation of the sympathetic vasodilator system (ELIASSON *et al.* 1951) No attempt was made, however to investigate the mechanism behind the cardiac acceleration.

Method

Successful experiments were performed on 32 cats weighing 1.9 to 4.2 kg and anesthetized with chloralose (25—50 mg/kg) and urethane (200—500 mg/kg) i. About 80 cats were originally prepared, but technical difficulties encountered in attachment of the strain gauge arch to the heart, as well as in the maintenance of adequate cardiovascular conditions throughout the experiment necessitated elimination of number of animals.

The trachea was cannulated. Arterial pressure was recorded by means of a plastic cannula inserted into one carotid artery with its tip in the aortic arch. The pressure was measured with a Statham transducer (P 23 A) Rectal temperature was maintained at about 37° C by means of an infrared lamp directed toward the animal' thorax and abdomen.

Blood flow was directed to a silicone-filled drop chamber (LINDGREN 1958) and via photocell the drop rate was recorded by an ordinate writer Muscle blood flow was determined in the right femoral artery in skinned hind limb. For exclusion of paw blood flow tight ligature was applied immediately bove the ankle. T maintain warmth and moistness, the skin was replaced around the muscle In 3 experiments the blood flow to an intestinal area was measured in branch of the superior mesenteri artery T prevent clotting, heparin (Heparin 5 %' Vitrum) 25 mg/kg was administered i.

In 11 of the experiments the cats were initially subjected to bilateral cervical vagotomy I 3 of these, carotid occlusion was maintained to avoid possible cardiac changes due to baroceptor mechanisms. Dextran (Macrodex, Pharmacia) was given v as required, to compensate for blood loss. Positive pressure artificial respiration was maintained during the experiments.

Central stimulation was effected by the Horsley-Clarke technique. With use of a dental drill the skull was trephined for insertion of the electrode into the right side of the hypothalamus. Electrical stimulation (2.0—2.5 V 70 cps and pulse duration 2 msec with insulated stainless steel unipolar electrodes about 0 45 mm thick and with an uninsulated pointed tip 0.5 mm long) was produced by a square w ve generator

The tw stellate ganglia were carefully located via bilateral approach through the first costal interspace. The chest wall was then closed. Subsequently t was opened for removal of the ganglia and reclosed

The two adrenals were dissected free via an abdominal pproach and later ligated and removed In some experiments, th pulmonary artery and either the bdominal aorta just below its passage through the diaphragm, or a thoracic part of the aorta, were mobilized and heavy thread placed loosely around each of them for subsequent stricture.

The right intraventricular pressure was recorded in 11 experiments with a Statham transducer (P 23 A) Following thoracotomy polyethylene catheter was advanced through the ventricular wall into the cavity with the aid of sharp metal guide which was then removed.

Heart rate was determined with an interval recorder (GOLDSCHMIDT and LINDGREN 1961) via impulses from the blood-pressure channel of Grass (Polygraph) instrument.

Strain Gauge Arch Recording of Cardiac Contractile Force

Cardiac contractile force was measured by strain gauge arch. In the following the terms "contractile force and "force of cardiac contraction insofar as they concern personal observations, denote the force of ventricular contraction as measured by a strain gauge arch.

After thoracotomy and opening of the pericardium the strain gauge arch was attached to the ventricular wall by sutures penetrating deeply into the muscle. All sutures were so placed as to avoid damage to or occlusion of any major branch of coronary vessel. The muscle segment between the two points of attachment was conventionally stretched 40—50 per cent, as described by COTTEN and BAY (1956) to minimize errors in the recording of any subsequent increase in the size of the ventricle. The absence of irregularities in the cardiac recordings after attachment of the arch indicated that the conducting system of the heart had not been traumatized. Care was taken to shield the opening in

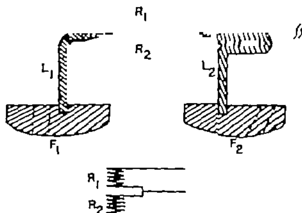


Fig 1 Sagittal section of modified strain gauge arch.
R and R_2 The strain gauge resistances.
L_1 and L_2 The legs of the arch. The distance between L_1 and L_2 is 9 mm.
F and F The feet with holes for sutures.

the chest wall, thus preventing drying and cooling of the heart. The intrathoracic pressure was atmospheric during the experiment.

In all but one of the 20 experiments in which the hypothalamus was stimulated, strain gauge arch recording of contractile force was done from the right ventricle In one, the arch was ttached to the left ventricle. Right ventricular contractile force was also recorded in 12 supplementary experiments designed for qualitative testing of the strain gauge arch technique. In 8 of these experiments left ventricular force was studied simultaneously

In 7 of the experiments in which the hypothalamus was stimulated, open strain gauge arches essentially similar to that reported by BONIFACE, BRODIE and WALTON (1953) were used. In the other experiments the strain gauge arch was slightly modified (Fig 1) and resulted in a more sensitive and stable recording Instead of one, two identical strain gauge wires (Philips PR 9214) each with a resistance of 120 ohms, were attached — one on either side of the metal (phosphor-bronze) strip connecting the two legs. With this arrangement, opposite changes occurred in the two resistances on application of a force between the two points of attachment of the arch the resistance increased in the elongated wire and decreased in the contracted one. To ensure stable fixation and sufficient insulation of the strain gauge wires, the metal strip was lengthened on one sid and attached to the subjacent part. By measuring the difference of the two resistances greater accuracy was achieved than by use of single strain gauge wire (Fig 2)

Supplementary Studies and Comments on the Qualifications of the Strain Gauge Arch Technique

In using the strain gauge arch technique for determination of cardia contractile force, it is necessary to bear in mind that changes of heart size may cause interference. It is therefore essential in the present experiments t be on the alert for any rise of the heart volume due to elevated pressure the aorta or to increased venous return.

Strain gauge arch recording of the left ventricular contractile force is influenced by the aortic pressure when the latter exceeds a certain level (Fig 3) This level may be reached on intracerebral stimulation. The rise of left ventricular diastolic pressure secondary to that of the aortic diastolic pressure tends t increase the diastolic length of the muscle fibers, which thus contract more vigorously Since the muscle segment between

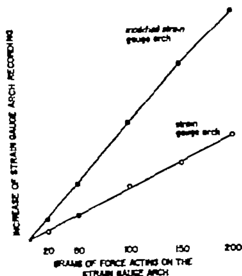


Fig. 2. Relationship between grams of force acting on the modified strain gauge arch and percentual increase f recorded force. Comparison with the sensitivity of the strain gauge arch described by Boniface *et al.* (1953) Under the experimental conditions the re corded force of the heart never exceeded 150 g.

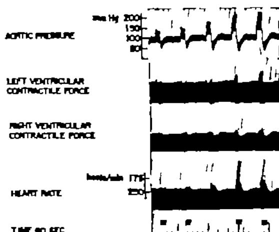


Fig 3. Left and right ventricular contractile force and heart rate responses to gradual increase f mechanical aortic stricture few millimeters distal to the origin of the left subclavian artery
Note the difference in response of left and right ventricle.

the two points of attachment of the strain gauge arch is already stretched, the length of the segment remains unaffected by moderate changes in heart size Certain fibers, however pass through only one point of attachment, though relatively parallel to the long axis of the arch. With enlargement of the heart these fibers would be lengthened and would accordingly contract more vigorously (Cotten and Bay 1956) thus accounting for cases with increased strain gauge arch recording on heart enlargement. I the only experiment (cat no. 9) which involved hypothalamic stimulation in connection with left ventricular attachment of the strain gauge arch, the rise of mean aortic pressure during central stimulation was so low (15 mm Hg) that its effect on the recording of left ventricular contractile force was insignificant. This observation accords with data from similar experiments performed by Cotten and Bay

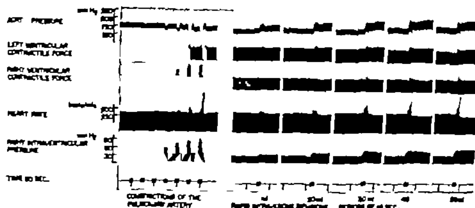


Fig. 4. Left and right ventricular contractile force, right intraventricular pressure, and heart rate responses to a gradual increase of mechanical stricture of the pulmonary artery and to rapid intravenous blood and/or dextran infusions.
Note the slight increases of contractile force, if any, with rapid intravenous infusion. The weight of the cat was 2.7 kg.

The effect produced on the strain gauge arch recording of left ventricular contractile force by a rise in aortic pressure due to artificial constriction of the thoracic aorta, was studied in 4 experiments (one of which is illustrated in Fig. 3). Results similar to those found in the experiment in Fig. 3 were obtained.

The influence of increased aortic pressure on the strain gauge arch recording from the right ventricle is insignificant, as illustrated in Fig. 3. This fact accords with the results obtained in the other 12 experiments in which the abdominal or the thoracic aorta was artificially constricted. In each of these experiments the rise of mean aortic pressure amounted to at least 50 per cent of resting level.

Augmentation of venous return by rapid intravenous infusion of dextran influences the strain gauge arch recording of the right ventricle to only a slight degree (COTTEN 1953) as was confirmed in the present study. In 7 experiments in which a cannula was inserted into a jugular vein and the tip placed close to the entrance to the right atrium, infusion of blood and/or dextran in amounts up to 4—7 ml per second over periods of 5—10 sec, caused only a slight increase in the strain gauge arch recording. The increase is insignificant as compared to the relatively substantial augmentation caused by hypothalamic activation of the inotropic fibers to the heart. The response to gradual increase of venous return as well as to artificial constriction of the pulmonary artery in the experiment illustrated in Fig. 4 is in line with the effects obtained in the other six experiments. The cardiac output in a cat of 2.5 kg is reported to be in the region of 270 ml per minute (SPECTOR 1956). Assuming that the resting cardiac output in my experiments amounted to 250—400 ml, the venous return to the heart must have reached almost double that value in the infusion experiments. The maximal increase of venous return which may, in the relevant experiments, be elicited by the fairly mild central stimulation of the sympathetic vasodilator outflow is probably of the same magnitude as that produced by the infusions.

Because of the above considerations and for the sake of a convenient operative approach, the right ventricle was considered preferable to the left in experiments in which hypothalamic stimulation was applied in conjunction with measurement of cardiac contractile force.

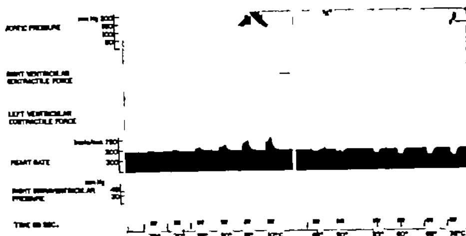


Fig. 5. Left and right ventricular contractile force and right intraventricular pressure responses to alterations of the heart rate produced by topical application of saline of varying temperature on the right atrium.
Note the minimal responses of contractile force to the varying heart rates.

That the effect of heart-rate changes on the strain gauge arch recording of contractile force is negligible (COTTEN 1953) was confirmed in 6 of my experiments. In 3 of these pacemaker with the two electrodes separated by 3 mm was attached to the right atrium and stimulated with various frequencies (2.0 V and pulse duration 1.5 msec) in the other three, saline solution of varying temperature (Fig. 5) was topically applied to the region of the right atrium where the sinu-atrial node is situated, and produced alterations of heart rate. In each of the experiments an increase of 20 to 70 per cent in the range of 150—300 beats per minute was evoked with insignificant responses in the strain gauge arch recording of cardiac contractile force.

Results

Cardiac Responses to Hypothalamic Stimulation Producing Muscle Vasodilatation

In the 18 experiments in which skeletal muscle vasodilatation was produced by hypothalamic stimulation, cardiac contractile force and heart rate were continuously recorded. Fig 6 illustrates a typical experiment. "Hyp I in the figure indicates a response to stimulation in the anterior part of the hypothalamus whereby the sympathetic vasodilator outflow was activated After a latency of 4 sec or less, muscle vasodilatation, tachycardia and an increase of right ventricular contractile force appeared. The short latency of the responses was consistent with that in all except two of the other experiments the two exceptions will be discussed later on. Intravenous injection of atropin 0 3 mg/kg blocked the muscle vasodilator response to stimulation, a fact suggestive of a true cholinergic effect. In all experiments in which atropine was applied, the muscle vasodilator response was either markedly depressed or abolished. The cardiac responses were not affected by atropine either in the experiment illustrated in Fig 6, or in the other experiments, but almost disappeared on removal

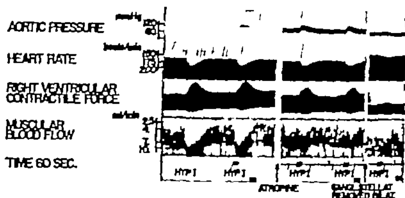


Fig. 6. Cat 17 Cardiac contractile force, heart rate and muscle blood flow responses to stimulation in the hypothalamus before and after atropinization and removal of the two stellate ganglia. The adrenals were intact.
HYP I Hypothalamic stimulation 2.0 V 70 cps.
Atropine 0.3 mg/kg intravenously
The figures indicate the elapsed time in minutes after the completion of preparation.
Note that there is no change in muscle blood flow on stimulation after atropine and that the cardiac responses almost disappear following removal of the two stellate ganglia.

of the two stellate ganglia. As will be seen in the figure, stellatectomy in itself caused a reduction of about 50 per cent in the contractile force. A similar reduction was produced in some of the other experiments, but more often the decrease was less. In 9 experiments in which right intraventricular pressure was measured, stellatectomy caused a slight reduction of the systolic pressure concomitantly with the decrease in contractile force (Rosén 1961 a)

In all experiments cardiac augmentation accompanied the muscle vasodilator response following hypothalamic stimulation (see Table I and II) The short latency of cardiac responses to hypothalamic stimulation suggests that nervous mechanisms are implicated. Evidence of this was found both in the experiment illustrated in Fig 6 and in others (Table II) in which removal of the two stellate ganglia caused a marked depression or abolition of the cardiac responses.

In 2 experiments (cat 12 and 13 in Table II) carotid occlusion was produced in the interval between two identical hypothalamic stimulations and was maintained during the second stimulation. The magnitude of the cardiac response was the same after each stimulation, being unaffected by the persistent carotid occlusion. In one experiment (cat 6 in Table I) carotid occlusion had been done before any hypothalamic stimulation was carried out. Bilateral vagotomy was performed in seven experiments prior to hypothalamic stimulation.

In 3 experiments (cat 2 9 and 17 in Table I and II) the intestinal blood flow was recorded during central stimulation of the sympathetic vasodilator pathway Vasoconstriction occurred in each case, a fact which accorded with earlier observations (Eliasson et al 1951)

Table I Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to hypothalamic stimulation of the sympathetic vasodilator pathway eliciting muscle vasodilator response

Cat. no.		C. F	H. R.
1	A	+++	++
2	A	++	not measured
3		++	0
4		+++	not measured
5		++	++
6	V PCO	++	++
7		+++	++
8		+++	+

A = Adrenalectomized
V = Vagotomized
PCO = Persistent carotid occlusion

C.F +++ correspond to the response of about 1.0 μg/kg adrenaline i.v.
++ correspond to the response of about 0.5 μg/kg adrenaline i.v.
\+ correspond to the response of about 0.2 μg/kg adrenaline i.v.
0 Insignificant response

H.R. +++ 15—20 % increase
++ 10—15 % increase
\+ 5—10 % increase
0 < 5 % increase (insignificant response)

Table II Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to hypothalamic stimulation of the sympathetic vasodilator pathway (eliciting muscle vasodilator response) following adrenalectomy and/or stellatectomy

Cat. no.		C. F	H. R.	After adrenalectomy C. F	After adrenalectomy H. R.	After stellatectomy C. F	After stellatectomy H. R.
9		++	+	+	0		
10	V	+++	+	0	0		
11	V S	+++	0	0	0		
12	V PCO	+++	+	+++	+	0	0
13	V PCO	+++	++	+	+	0	0

Cat. no.		C. F	H. R.	After stellatectomy C. F	After stellatectomy H. R.	After adrenalectomy C. F	After adrenalectomy H. R.
14	A	++	++	0	0		
15	V	+++	++	0	0		
16		+++	+	+	+		
17		++	+++	0	+	0	0
18	V	++	+++	+	++	0	+

S = Stellectomized
A, V PCO, C. F (+++ ++ + 0) and H. R. (+++ ++ + 0) see table I

The Magnitude of Increased Cardiac Contractile Force and Heart Rate

In all experiments the increase in cardiac contractile force following hypothalamic stimulation of the sympathetic vasodilator pathway was estimated by determining the dose of adrenaline required to produce, via intravenous injection, a like increase in the force of contraction. The adrenaline was injected not long before or after the hypothalamic stimulation to insure that the cardiac contractility conditions would not differ from those under the hypothalamic activation.

Difficulties were encountered in making a percentual calculation of the observed increases of cardiac contractile force, since the cardiac responses to identical repeated hypothalamic stimulation sometimes varied substantially in a given experiment, particularly when the intervals between stimuli were protracted. In these experiments it was found, however that the responses varied quantitatively in the same way as those produced by intravenous injection of adrenaline.

The increase of cardiac contractile force accompanying vasodilatation in skeletal muscle on stimulation of the sympathetic vasodilator outflow was, under the present experimental conditions, of the same magnitude as that following intravenous injection of 0.5 to 1.0 µg adrenaline per kg (see Table I and II) The heart acceleration, as will be seen from Table I and II generally amounted to between 5 and 20 per cent.

Following removal of the stellate ganglia, and likewise after adrenalectomy adrenaline was always injected once more for estimation of any augmentation of the contractile force with further hypothalamic stimulation. The effect of adrenaline on the contractile force was always at least as pronounced after blocking of the sympathetic cardiac innervation as it had been before (Fig 7)

Influence of Catechol Release from the Adrenals on Cardiac Responses and on Muscle Vasomotor Responses

In 8 experiments the influence of catechol output from the adrenals on cardiac responses following hypothalamic stimulation of the sympathetic vasodilator pathway was determined (Table II) In three the original response of cardiac contractile force was partly inhibited after adrenalectomy In a further two (cat 10 and 11) the response was abolished. The latter finding suggests that the effects were elicited entirely by released catechols — a possibility which is supported by the relatively long latency (10—12 sec) of the cardiac responses in these two experiments. The muscle vasodilator response appeared, however within 4 sec after the onset of stimulation in these two experiments. The heart acceleration was reduced or abolished in 5 experiments by adrenalectomy

In two or three of the five cats in which stimulation caused a catechol release from the adrenals of sufficient magnitude to influence the force of cardiac contraction, the adrenaline output may even have sufficed to produce slight muscle vasodilatation.

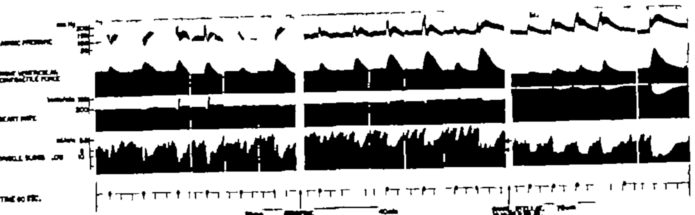


Fig. 7 Cat 16. Cardiac contractile force, heart rat and muscle blood flow responses t stimulation t various levels i the hypothalamus before and after atropinization and removal of the two stellate ganglia. The adrenals were intact.

1—5 Hypothalamic stimulation (2 0 V 70 cps) delivered at five horizontal levels approximately 1 mm apart while the electrode was gradually shifted ventrally

A. Adrenaline 1.0 μg/kg intravenously

Atropine 0 5 mg/kg intravenously

The figures minutes indicate the elapsed time after the completion of preparation.

Note that the muscle vasodilator responses appear concomitantly with an increase of cardiac contractile force during ventral shifting of the electrode

Histologic Examination

Fifteen brains were examined histologically After removal they were fixed in 10 per cent formaldehyde solution Frozen serial sections were cut in a plane parallel to the electrode position.

It was found that all localized points the stimulation of which had elicited vasodilatation in skeletal muscle and concomitant cardiac augmentation, ere situated within the hypothalamic vasodilator region reported by LINDGREN *et al.* (1956) In two experiments muscle vasoconstriction occurred concomitantly with cardiac augmentation upon hypothalamic stimulation. The stimulated points were, however found to be situated within the region through which the vasodilator pathway passes (Fig 9) In Fig 9 and 10, sections of the hypothalamus show the 13 localized points which when stimulated, were associated with muscle vasodilatation and increased contractile force.

Discussion

Recording of cardiac contractile force is readily accomplished with a strain gauge arch — a method which Cotten and coworkers have shown to be exceptionally sensitive for direct measurement of acute changes in the contractility Its validity has been further substantiated in the present experiments For reasons reported in the foregoing, it was found appropriate to record the contractile force from the right ventricular muscle.

The strain gauge arch technique is highly serviceable for experiments in which no substantial changes occur in the size of the heart (COTTEN and BAY 1956) as has been confirmed in the present investigation. Since the muscle segment between the two points of attachment of the strain gauge arch is stretched, the tracing is not affected to any noticeable extent by the occurrence of a moderate heart enlargement, though it may be appreciably influenced by a more pronounced heart enlargement. If hypothalamic stimulation produced an enlargement of the right ventricle, the change in ventricular size was probably insufficient to influence noticeably the strain gauge arch recording This was demonstrated by studying the degree to which factors determining the heart volume influenced the strain gauge arch recording from the right ventricle. The tracing was not noticeably affected by an increase either n aortic pressure or in the enous return to the heart, of magnitudes such as could result from the relevant hypothalamic stimulation. Cardiac acceleration does not significantly influence the strain gauge arch recording (COTTEN 1953) as was confirmed in my investigations.

Recent studies ha e demonstrated the presence, in the sympathetic outflow of fibers which affect the contractile force of the heart (COTTEN 1953, ANZOLA and RUSHMER 1956 RANDALL and ROHSE 1956) Stimulation of these fibers increases the contractile force without necessarily raising the heart rate (RANDALL and ROHSE 1956 The central representation of these inotropic fibers to

the heart is obscure, though RUSHMER *et al.* (1959) and SMITH *et al.* (1960) have demonstrated that the left ventricular stroke work is increased by stimulation of a few circumscribed areas in the hypothalamus. MANNING and PEISS (1960) have drawn, solely on the basis of blood pressure changes, conclusions regarding augmentation of the cardiac contractile force. They observed increases of the "contractile force" on stimulating large areas of the hypothalamus. — As regards the central representation of inotropic fibers to the heart, my investigations have confirmed the Rushmer group's observations and have also demonstrated the presence of a single, relatively well-circumscribed area, extending from the anterior to the posterior hypothalamus, the stimulation of which increases the cardiac contractile force. Under the relevant experimental conditions the augmentation was equivalent to that produced by intravenous injection of adrenaline 0.5—1.0 μg/kg.

It has long been known that various hypothalamic structures can, when activated, influence the heart rate. RUSHMER and his co-workers, as well as MANNING and PEISS, frequently observed elevation of the heart rate as a result of hypothalamic stimulation. It was found in my investigation that tachycardia generally accompanied the increase of contractile force elicited by the relevant stimulation in the hypothalamus. Under the prevailing experimental conditions the increase in heart rate amounted to a maximum of 20 per cent.

Those areas the stimulation of which produced an increase of cardiac contractile force and usually tachycardia, appeared to coincide in localization with the sympathetic vasodilator outflow which passes through the hypothalamus (LINDGREN *et al.* 1956). When vasodilatation was elicited in skeletal muscle by stimulation of the sympathetic vasodilator pathway it was invariably accompanied by cardiac augmentation. However, in 2 of the 20 experiments in which hypothalamic stimulation was applied a moderate muscle vasoconstrictor response appeared concomitantly with an increased cardiac contractile force. The histologic localization of the two stimulation points coincided with other stimulation points located within the area through which the sympathetic vasodilator pathway passes. It is possible therefore, that in these experiments skeletal muscle vasodilatation was concealed by vasoconstriction. ELIASSON *et al.* (1951) observed that vasoconstrictor effects could occur in the muscle simultaneously with vasodilatation on central stimulation of the vasodilator pathway.

In general the cardiac effects were either partly or entirely mediated by the sympathetic nerves, since in almost all experiments (18 out of 20) the latency was 4 sec or less. Also removal of the stellate ganglia in those animals with the short latency reduced or abolished the responses. The role of the adrenal medulla in the cardiac responses varied. In two experiments adrenalectomy abolished the cardiac responses, in others there was either partial reduction or no effect. GRANT *et al.* (1958) reported that under their experimental conditions, one adrenal gland releases on the average 0.32 μg/kg/min adrenaline and 0.35

The Magnitude of Increased Cardiac Contractile Force and Heart Rate

In all experiments the increase in cardiac contractile force following hypothalamic stimulation of the sympathetic vasodilator pathway was estimated by determining the dose of adrenaline required to produce via intravenous injection, a like increase in the force of contraction. The adrenaline was injected not long before or after the hypothalamic stimulation to insure that the cardiac contractility conditions would not differ from those under the hypothalamic activation.

Difficulties were encountered in making a percentual calculation of the observed increases of cardiac contractile force, since the cardiac responses to identical repeated hypothalamic stimulation sometimes varied substantially in a given experiment, particularly when the intervals between stimuli were protracted. In these experiments it was found, however that the responses varied quantitatively in the same way as those produced by intravenous injection of adrenaline.

The increase of cardiac contractile force accompanying vasodilatation in skeletal muscle on stimulation of the sympathetic vasodilator outflow was, under the present experimental conditions, of the same magnitude as that following intravenous injection of 0.5 to 1.0 μg adrenaline per kg (see Table I and II). The heart acceleration, as will be seen from Table I and II generally amounted to between 5 and 20 per cent.

Following removal of the stellate ganglia, and likewise after adrenalectomy adrenaline was always injected once more for estimation of any augmentation of the contractile force with further hypothalamic stimulation. The effect of adrenaline on the contractile force was always at least as pronounced after blocking of the sympathetic cardiac innervation as it had been before (Fig 7)

Influence of Catechol Release from the Adrenals on Cardiac Responses and on Muscle Vasomotor Responses

In 8 experiments the influence of catechol output from the adrenals on cardiac responses following hypothalamic stimulation of the sympathetic vasodilator pathway was determined (Table II). In three the original response of cardiac contractile force was partly inhibited after adrenalectomy. In a further two (cat 10 and 11) the response was abolished. The latter finding suggests that the effects were elicited entirely by released catechols — a possibility which is supported by the relatively long latency (10—12 sec) of the cardiac responses in these two experiments. The muscle vasodilator response appeared, however within 4 sec after the onset of stimulation in these two experiments. The heart acceleration was reduced or abolished in 5 experiments by adrenalectomy.

In two or three of the five cats in which stimulation caused a catechol release from the adrenals of sufficient magnitude to influence the force of cardiac contraction, the adrenaline output may even have sufficed to produce slight muscle vasodilatation.

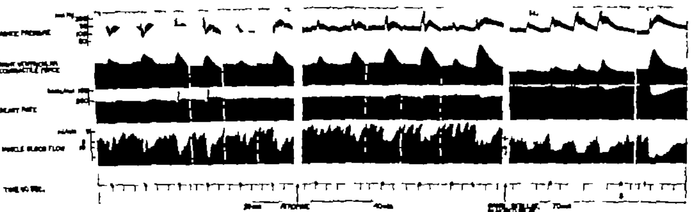


Fig. 7 Cat 16. Cardiac contractile force, heart rate and muscle blood flow responses to stimulation at various levels in the hypothalamus before and after atropinization and removal of the two stellate ganglia. The adrenals were intact.

1—5 Hypothalamic stimulation (2.0 V 70 cps) delivered at five horizontal levels approximately 1 mm apart while the electrode was gradually shifted ventrally

A. Adrenaline 1.0 μg/kg intravenously

Atropine 0.5 mg/kg intravenously.

The figures in minutes indicate the elapsed time after the completion of preparation.

Note that the muscle vasodilator responses appear concomitantly with an increase of cardiac contractile force during ventral shifting of the electrode.

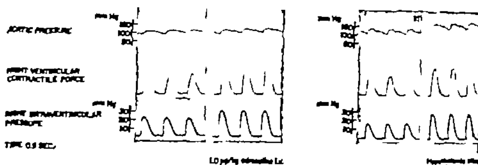


Fig. 8. Cat 19 Tracings of right ventricular contractile force and right intraventricular pressure following injection of adrenaline and during hypothalamic stimulation.

Cardiac Responses to Hypothalamic Stimulation Eliciting No Muscle Vasodilatation

During attempts to locate hypothalamic points the stimulation of which would elicit muscle vasodilatation, two methods were performed. In one, the electrodes were moved ventrally with continuous stimulation until a response was obtained. In the other (8 experiments) the electrodes were moved ventrally by one mm steps, stimulation being applied at each point. In all experiments a muscle vasodilator response apparently occurred at the same horizontal level as did cardiac augmentation. Fig 7 illustrates an experiment in which identical stimuli were delivered at various horizontal levels, the numerals 1 to 5 indicating levels approximately one mm apart. Each muscle vasodilator response elicited by stimulation was accompanied by a cardiac augmentative response.

In 2 of the 20 experiments (cat 19 and 20) in which hypothalamic stimulation was applied, moderate muscle vasoconstriction was observed however concomitantly with increased force of cardiac contraction. The increase of contractile force and heart rate in these two experiments was equivalent to that in the experiments in which muscle vasodilatation accompanied the cardiac response and appeared, in addition, within 4 sec after the onset of stimulation.

Simultaneous Recording of Right Ventricular Contractile Force and Right Intraventricular Pressure

Alterations in the duration of the isometric pressure gradient as well as other changes of the pulse contour may serve as criteria of changes in the force of ventricular contraction.

Right intraventricular pressure was measured, in four experiments (cat 3, 10, 12 and 19) concurrently with strain gauge arch recording of contractile force from the right ventricle. One experiment is illustrated in Fig 8. The pulse contour of the intraventricular pressure was compared with the strain gauge arch recording after injection of 1.0 μg adrenaline per kg and during hypothalamic stimulation. The tracing was made a few seconds after the

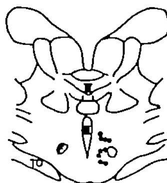


Fig. 9. Drawing of frontal section through cat hypothalamus 3—4 mm posterior to the anterior commissure.

Stimulated points, histologically localized to region 1.5 mm anterior or posterior to the section, are represented by the black and the dotted white circles. All points were associated with increased cardiac contractile force when stimulated.

(F fornix TO optic tract; III third ventricle.)

● Cardiac augmentation and muscle vasodilatation

◐ Cardiac augmentation and muscle vasoconstriction

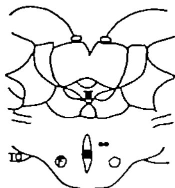


Fig. 10. Drawing of frontal section through cat hypothalamus 7—8 mm posterior to the anterior commissure. Stimulated points, histologically localized to region 1.5 mm anterior or posterior t the section, are represented by the black circles. All points were associated with increased cardiac contractile force and muscle vasodilatation when stimulated.

(F TO and III, see text to Fig. 9)

onset of increase in amplitude of the strain gauge arch record ng Both adrenaline and hypothalamic stimulation increased the contractile force as measured by the strain gauge arch. Concomitantly the pulse contour revealed a diminution in duration of the isometric pressure gradient, an accelerated rise in tension, increased systolic pressure, and more rapid relaxation. In the other 3 experiments, intraventricular pressure responses of the same character were obtained. In none of the 4 experiments was the rise in heart rat sufficient to account for the decreased duration of the isometric pressure gradient. A change of diastolic pressure in the pulmonary artery causing the decreased duration response probably did not occur The bserved changes of pressure conform to parameters which other uthors consider t be indicative of increased force of cardiac contraction during stimulation of th stellate ganglia (ARZOLA and RUSHMER 1956, KELSO and RANDALL 1959) The present results are in agreement with those found by COTTEN and BAY (1956) in their investigation of the action of cardioacti e drugs on the strain gauge arch recording and on the duration of the isometric pressure gradient.

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μg/kg/min noradrenaline upon hypothalamic stimulation in the sympathetic vasodilator area. Intravenous adrenaline or noradrenaline infusion of 0.1—0.5 μg/kg/min is considered to be sufficient to cause an increase of cardiac contractile force (Rosén 1961 b). Since the present experimental conditions are similar to those of Grant *et al.* and of Rosén it is not surprising to find that cardiac augmentation partly due to catechols released from the adrenals, occurs when the vasodilator outflow is activated in the hypothalamus.

The present experiments showed that cardiac augmentation probably resulting in an increased output, as well as skeletal muscle vasodilatation splanchnic vasoconstriction, and catechol discharge from the adrenals were characteristic responses to hypothalamic stimulation of the vasodilator system. The cardioactive fibers found in this investigation were associated with the 'vasodilator area since it was not possible to demonstrate them immediately outside it. The question arises whether the concurrence of these responses is purely coincidental or whether it points to a functional entity. To judge from the results, the hypothesis *of a functional entity is plausible. Such an assumption is further supported* by the observation that an increased force of cardiac contraction as well as tachycardia occur on stimulation of the vasodilator pathway in the oblongate medulla (Rosén 1961 a).

There are several conditions associated with a cardiovascular pattern of the same type as that obtained by hypothalamic activation of the sympathetic vasodilator system. One such condition is emotional excitement. Increased cardiac output (Stead *et al.* 1945, Hickam, Cargill and Golden 1948) vasodilatation in skeletal muscle (Blair *et al.* 1959) and increased catechol release, mainly of adrenaline, from the adrenals (for references see Euler 1956) are all relevant to emotional stress. Furthermore, in this condition the hypothalamus is known to play an important integrative role. That the sympathetic vasodilator pathway may be activated during conditions of emergency was suggested by Abrahams and Hilton (1958). They stimulated the hypothalamic vasodilator pathway in anesthetized cats and allowed the animals to recover consciousness with the electrode *in situ*. Repeated stimulation produced responses described as "flight and fight reactions". In another series of experiments on conscious cats Abrahams *et al.* (1960a) observed, following acoustic and nociceptive stimulation, a rise of temperature in the venous blood from the skeletal muscle. They suggested that this response might be caused by muscle vasodilatation elicited by activation of the sympathetic vasodilator outflow. The vasodilator response was presumed to be part of a defence reaction.

The fact that impulses from the brain may produce cardiac augmentation, cardiac acceleration and muscle vasodilatation also leads to other speculations. Rushmer *et al.* (1959) pointed out that the presence of such mechanisms appears essential to an explanation of the very abrupt responses at the onset of exercise and of the cardiovascular adjustments occurring in anticipation of exercise. They found that changes of cardiac functions following hypothalamic stimula-

tion were distinctly similar to treadmill exercise responses. The fact that the sympathetic vasodilator outflow has a cortical origin (ELIASSON, LINDGREN and UVNÄS 1952) is one of the reasons for the inference that it may be involved in the initial phase of muscle exercise. The present finding that augmented cardiac contraction, tachycardia, and muscle vasodilatation occur concomitantly in response to hypothalamic stimulation supports the hypothesis that the vasodilator system may be concerned with the initial adjustment of the muscular blood flow during exercise.

The expenses of this investigation were defrayed by the Swedish Medical Research Council, the Swedish National Association against Heart and Chest Diseases and Karolinska Institutet.

References

ABRAHAMS, V. C. and S. M. HILTON, Active muscle vasodilatation and its relation to the 'Flight and Fight Reactions' in the conscious animal. *J. Physiol.* (Lond.) 1958. *140*. 16—17 P

ABRAHAMS, V. S., S. M. HILTON and A. ZBROŽYNA, Reflex activation of vasodilator nerve fibres to skeletal muscle in decerebrate and intact cats. *J. Physiol.* (Lond.) 1960 a. *152*. 54—55 P

ABRAHAMS, V. C., S. M. HILTON and A. ZBROŽYNA, Active muscle vasodilatation produced by stimulation of the brain stem: Its significance in the defence reaction. *J. Physiol.* (Lond.) 1960 b. *154* 491—513.

ANDOLA, J. and R. F. RUSHMER, Cardiac responses to sympathetic stimulation. *Circulation Res.* 1956. *4* 302—307

BARCROFT H., O. EDHOLM, J. MCMICHAEL and E. P. SHARPEY-SCHAFER, Posthaemorrhagic fainting Study by cardiac output and forearm flow *Lancet* 1944 *1* 489—490.

BARCROFT H. and O. G. EDHOLM, On the vasodilatation in human skeletal muscle during post haemorrhagic fainting *J. Physiol.* (Lond.) 1945 *104* 161—173.

BLAIR, D. A., W. E. GLOVER, A. D. M. GREENFIELD and I. C. RODDIE, The activation of cholinergic vasodilator nerves in the human forearm during emotional stress. *J. Physiol.* (Lond.) 1959 *147* 27 P

BONIFACE, K. J., O. J. BRODIE and R. P. WALTON, Resistance strain gauge arches for direct measurement of heart contractile force in animals. *Proc. Soc. exp. Biol.* (N. Y.) 1953. *84* 263—266.

COTTEN, M. DE V. Circulatory changes affecting measurement of heart force in situ with strain gauge arches. *Amer. J. Physiol.* 1953. *174* 365—370.

COTTEN, M. DE V. and E. BAY Direct measurement of changes in cardiac contractile force. *Amer. J. Physiol.* 1956. *187* 122—134

ELIASSON, S., B. FOLKOW, P. LINDGREN and B. UVNÄS, Activation of sympathetic vasodilator nerves to the skeletal muscles in the cat by hypothalamic stimulation. *Acta physiol. scand.* 1951 *23*. 333—351

ELIASSON, S., P. LINDGREN and B. UVNÄS, Representation in the hypothalamus and the motor cortex in the dog of the sympathetic vasodilator outflow to the skeletal muscles *Acta physiol. scand.* 1952. *27* 18—37

EULER, U. S. Noradrenaline. Springfield. Ill. U. S. A. 1956.

GOLDSCHMIDT H. and P. LINDGREN An electronic interval recorder for measuring peripheral blood flow and heart rate. *J. appl. Physiol.* 1961 T. be published.

GRANT R., P. LINDGREN, A. ROSÉN and B. UVNÄS, The release of catechols from the adrenal medulla on activation of the sympathetic vasodilator nerves to the skeletal muscles in the cat by hypothalamic stimulation. *Acta physiol. scand.* 1958. *43*. 135—154.

HICKAM, J. B., W. H. CARGILL and A. GOLDEN, Cardiovascular reactions to emotional stimuli. Effect on the cardiac output, arteriovenous oxygen difference, arterial pressure, and peripheral resistance. *J. clin. Invest.* 1948. *27.* 290—298.

KELSO, A. F. and W. C. RANDALL, Ventricular changes associated with sympathetic augmentation of cardiovascular pressure pulses. *Amer. J. Physiol.* 1959. *196.* 731—734.

LINDGREN, P., The mesencephalon and the vasomotor system. *Acta physiol. scand.* 1955. *35.* Suppl. 121. 1—189.

LINDGREN, P., An improved method for drop recording of arterial or venous blood flow. *Acta physiol. scand.* 1958. *42.* 5—11.

LINDGREN, P., A. ROSÉN, P. STRANDBERG and B. UVNÄS, The sympathetic vasodilator outflow — a cortico-spinal autonomic pathway. *J. comp. Neurol.* 1956. *105.* 95—109.

LINDGREN, P., A. ROSÉN and B. UVNÄS, The release of catechols from the adrenal medulla on activation of the bulbar part of the sympathetic vasodilator outflow in cats. *Acta physiol. scand.* 1959 a. *47.* 233—242.

LINDGREN, P., A. ROSÉN and B. UVNÄS, Quantitative aspects of the vasomotor action of adrenaline and noradrenaline in the cat. Relationship to catechol discharge on intracerebral stimulation of the sympathetic vasodilator outflow. *Acta physiol. scand.* 1959 b. *47.* 243—250.

LINDGREN, P. and B. UVNÄS, Activation of sympathetic vasodilator and vasoconstrictor neurons by electrical stimulation in the medulla of the dog and cat. *Circulation Res.* 1953. *1.* 479—485.

MANNING, J. W. and C. N. PEISS, Cardiovascular responses to electrical stimulation in the diencephalon. *Amer. J. Physiol.* 1960. *198.* 366—370.

RANDALL, W. C. and W. G. ROHSE, The augmentor action of the sympathetic cardiac nerves. *Circulation Res.* 1956. *4.* 470—475.

ROSÉN, A., Augmentation of cardiac contractile force and heart rate by medulla oblongata stimulation in the cat. *Acta physiol. scand.* 1961 a. To be published.

ROSÉN, A., Cardiac inotropic and skeletal muscle vasomotor responses to adrenaline and noradrenaline. Threshold doses on intravenous infusion. *Acta physiol. scand.* 1961 b. To be published.

RUSHMER, R. F., O. SMITH and D. FRANKLIN, Mechanisms of cardiac control in exercise. *Circulation Res.* 1959. *7.* 602—627.

SMITH, O. A. JR., S. J. JABBUR, R. F. RUSHMER and E. P. LASHER, Role of hypothalamic structures in cardiac control. *Physiol. Rev.* 1960. *40.* Suppl. 4. 136—141.

SPECTOR, W. S., Handbook of Biological Data. Saunders. Philadelphia and London. 1956.

STEAD, E. A. JR., J. V. WARREN, A. J. MERRILL and E. S. BRANNON, The cardiac output in male subjects as measured by the technique of right atrial catheterization. Normal values with observations on the effect of anxiety and tilting. *J. clin. Invest.* 1945. *24.* 326—331.

UVNÄS, B., Sympathetic vasodilator outflow. *Physiol. Rev.* 1954. *34.* 608—618.

Acta physiol. scand. 1961. 52. 309—319

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Further Observations on Anaphylactic Mast-Cell Changes in the Nasal Mucosa of Guinea Pigs in Vivo

By

LARS OLOF BORÉUS

Received 25 March 1961

Abstract

BORÉUS, L. O. *Further observations on anaphylactic mast-cell changes in the nasal mucosa of guinea pigs in vivo.* Acta physiol. scand. 1961. *52.* 309—319. — The anaphylactic reactions of tissue mast cells were quantitatively studied in the nasal mucosa of anaesthetized guinea pigs *in vivo*. Intra-arterial challenge with antigen produced extensive disappearance of the mast cells, as well as anaphylactic shock symptoms, in animals sensitized passively with rabbit anti-ovalbumin or actively by intranasal application of ovalbumin. ACTH and cortisone in high doses did not prevent the development of active anaphylaxis, nor did they significantly alter the average mast-cell population of the tissue. Moreover, the cell reaction to antigen was not antagonized by pretreatment with these hormones. Heating of the animal prior to challenge and prolongation of the injection time diminished the anaphylactic mast-cell reaction and the intensity of shock symptoms. The "missing" mast cells were seen to reappear in about 7 weeks. On administration of antigen *in vivo* they again displayed a strong disappearance reaction.

Administration of antigen to the nasal mucosa of sensitized guinea pigs *in vivo* causes a rapid and extensive decrease in the mast-cell count, which may be studied quantitatively (BORÉUS 1960). In this investigation the method was used to study the local anaphylactic mast-cell response under various experimental conditions, namely after passive sensitization of the animal with rabbit antiserum and after active sensitization by topical, intranasal application of egg albumin. In addition, the possible influence of ACTH and cortisone on the development of active anaphylaxis has been studied. Finally, observations relating to inhibition of the anaphylactic mast-cell disappearance and to the reappearance of previously abolished mast cells in the mucosa are reported.

Methods

Guinea pigs of both sexes were used.

Active sensitization was produced, unless otherwise stated, by one subcutaneous injection and, three days later one intraperitoneal injection of 100 mg crystalline egg albumin. Intra-arterial challenge was done in the anaesthetized animal via the common carotid artery with 1 ml of a 10 % w/v solution of egg albumin in saline. The injection time was 1 minute. Two specimens of nasal mucosa were removed from one side prior to the injection of antigen, and two specimens were taken from the contralateral side 5 minutes after the injection. The mast-cell populations on the two sides were estimated and compared. Anaphylactic "disappearance" of mast cells was expressed as a percentual decrease in relation to the control. The details of this method have been previously presented (Boréus 1960)

The anti-ovalbumin rabbit serum used for passive sensitization of the guinea pigs was obtained by heart puncture of a rabbit which had received several intravenous and intraperitoneal injections of egg albumin during the preceding 3 weeks. The same serum was used in all experiments. The strength of the antiserum was roughly estimated by means of a quantitative precipitation test, which disclosed a titer of 1 10,000.

Results

I. *Development of anaphylactic mast-cell sensitivity*

Passive sensitization. Previous work on guinea pig mast cells *in vivo* (Boréus 1960, 1960 a) had been done with actively sensitized guinea pigs. A few experiments were now performed to ascertain if a similar mast-cell reaction *in vivo* could be induced following passive sensitization.

Three animals were given 1 2 and 4 intraperitoneal injections, respectively of 1 ml rabbit anti-ovalbumin serum. The injections were given on consecutive days, and the animals were challenged on the day after the last injection. The resulting mast-cell disappearance was for 1 ml, 62 and 79 % for 2 ml, 86 and 95 % and for 4 ml, 86 and 95 %. It was noted that the anaphylactic shock symptoms were fairly mild in all animals despite the intensive mast-cell reaction. An increased respiratory rate was observed in one animal, and urination and defaecation in another but no signs of severe broncho-constriction appeared and no anaphylactic death occurred.

To test the duration of the passively induced anaphylactic state of the mast cells, 5 other normal guinea pigs were given 2 injections, each of 1 ml rabbit anti-ovalbumin serum, on two subsequent days. They were challenged with egg albumin 6 11 12 and 13 days after the first injection. The fifth animal was tested on day 15 with 1 ml rabbit serum intra-arterially The mast-cell response in these 5 animals is shown in Fig 1 The animal challenged at day 6 was the only one to suffer fatal shock the others showed no symptoms. The last animal tested with rabbit serum on day 15 reacted with intensive, fatal anaphylactic shock, indicating that it had developed active anaphylactic sensitivity to the serum. Control experiments had shown that similar serum injections in normal animals did not affect the mast cells.

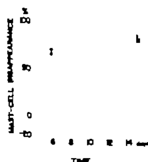


Fig 1 Anaphylactic mast-cell disappearance from nasal mucosa of guinea pigs at various intervals after passive sensitization with rabbit anti-ovalbumin serum. Challenge dose 100 mg egg albumin. T values for each animal are given. The hatched column denotes the mean of two disappearance values obtained in one animal upon challenge with 1 ml rabbit serum on day 15.

Active intranasal sensitization. Four normal guinea pigs were anaesthetized and placed in "hanging head position. By means of a thin, slightly bent steel cannula, 4 portions of 0.02 ml (2 mg) egg albumin were deposited, with 15-minute intervals, on the inferior nasal concha, the cannula being introduced through the left nostril. Great care was taken not to injure the mucosa. Except for some sneezing, the application produced no symptoms. In this way each animal received 8 mg egg albumin intranasally One, 3 5 and 7 days later the whole procedure was repeated, so that each animal received, in all, 40 mg egg albumin via the left nasal mucosa. (It should be pointed out, however that a part of that amount had been ejected from the nasal cavity by sneezing.) About 4 weeks later 3 of the animals were anaesthetized and challenged with antigen intravenously intra-arterially (left side) and intra-arterially (right side) respectively The fourth animal, however was sacrificed by a blow on the head and the nasal mucosa subjected to gross and microscopic examination. No injury of the mucosa was detectable and the mast cells on both sides appeared quite normal. The result of the challenge was as follows intravenous injection relatively severe anaphylactic shock with strong inspiratory movements, cyanosis and urination, accompanied by almost total disappearance of mast cells, on both the left and the right sides intra-arterial injection lethal shock with disappearance values 91 and 90 % (left side) and 99 and 96 % (right side)

In a later series of 6 guinea pigs a much smaller intranasal dose of egg albumin was shown to be sufficient to induce local and general anaphylactic sensitivity Two of the 6 animals were treated on days 1 2 and 4 as described above, each time with 8 mg egg albumin intranasally Two animals were treated on days 1 and 2, and two were treated once only on day 1 When challenged 3 weeks later with egg albumin the twice or thrice treated animals succumbed to anaphylactic shock. Th values for mast-cell disappearance in these animals ranged between 80 and 93 %. Of the two animals which received only a single treatment, one showed no shock symptoms when challenged, whereas the other evinced comparatively severe shock. The mean mast-cell disappearance was 43 %

Table I Dose schedule for ACTH cortisone and antigen during the sensitization period

		Day											
		1	2	3	4	5	6	7	8	9	10	11	12
Group 1													
Antigen	mg	100			100								
ACTH	IE	10	5	5	5	2.5	2.5	2.5	2.5	2.5	1.25	1.25	1.25
Group 2													
Antigen	mg	100			100								
Cortisone acetate	mg	25	12.5	12.5	12.5	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25

Influence of ACTH and cortisone on the development of active sensitization. Two groups of 5 guinea pigs each were sensitized in the usual way (one subcutaneous and, three days later one intraperitoneal injection of 100 mg egg albumin) those in the first group being simultaneously treated with ACTH and those in the second with cortisone. The dose schedule is given in Table I

On the thirteenth day the animals were challenged with an intra-arterial injection of 100 mg egg albumin. All of them reacted with fatal anaphylactic shock. The mean values for mast-cell disappearance were in the ACTH-treated group 78.4 ± 4.8 % and in the cortisone treated group 84 6 ± 2.8 %. These values did not differ significantly from the corresponding mean value for untreated, actively sensitized guinea pigs, challenged with like doses of antigen (cf. Boréus 1960) That treatment with these two hormones did not significantly reduce the mast-cell population of the nasal mucosa was established by comparing the values for the hormone-treated animals with 6 values for non-treated guinea pigs of the same stock. The values were ACTH-treated 3.2 ± 0 6, cortisone-treated 3 0 ± 0 3 and untreated controls 3 0 ± 0.6 cells per 0.256 mm

II *Modification of the mast-cell response to antigen*

Pretreatment with ACTH and cortisone Two actively sensitized guinea pigs received 10 IE ACTH subcutaneously and two received 25 mg cortisone acetate subcutaneously About 3 hours later they were challenged with an intra-arterial injection of 100 mg egg albumin. All four animals evinced fatal anaphylactic shock and an intense mast-cell reaction. The disappearance was (mean of 4 values each) in the ACTH-group 91.0 ± 2.3 % and in the cortisone-group 86.3 ± 9.8 %.

Heating of the animal. The effect of elevated temperature on the mast-cell sensitivity to antigen was tested by heating the anaesthetized tracheotomized guinea pig in a thermostatically regulated chamber (at 50—55 C) until the

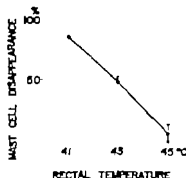


Fig. 2. Inhibition of anaphylactic mast-cell disappearance from the nasal mucosa of guinea pigs following heating of the animal. Challenge dose 100 mg egg albumin. Means and standard errors of 6 disappearance values.

desired rectal temperature had been attained (41—45 C). At that point, (reached after 30—40 minutes) the animal was removed from the box and the rectal temperature was allowed to fall to 38—39 C. The animal was then challenged intra-arterially in the usual way. The inhibitory effect of heating is evident from Fig. 2. The heating procedure was usually accompanied by some degree of respiratory and circulatory depression, though anaphylactic symptoms were still discernible in all three of the animals heated to 41 C as well as in one of those heated to 43 C. No shock symptoms could be detected in the other animals.

Variation of injection time. Twenty-five mg of antigen was administered intra-arterially to sensitized guinea pigs. The injection volume was in each instance 1 ml, but the injection time varied: in three animals it was 1 minute; in four 10 minutes; and in three, 30 minutes. A constant injection rate was assured by use of an infusion pump.

Preliminary experiments had exposed one drawback to this type of study i. e. the fact that some animals developed severe, often fatal anaphylactic shock within the 10-minute or 30-minute period. Thus, as a precaution, all animals in this series were pretreated with 1.25 mg promethazine, administered intraperitoneally about one half hour before challenge. Previous experiments had shown that this antihistamine treatment did not significantly influence the anaphylactic mast-cell disappearance in nasal mucosa but sufficed to protect the animals from symptoms of anaphylactic shock.

The disappearance values were as follows: 1 minute, 92.0 ± 2.7 %; 10 minutes, 78.8 ± 4.5 ; and 30 minutes, 22.7 ± 13.9 %. Thus, the standard error is higher in the 30-minute group than in the others. Since these animals were pretreated with an antihistaminic drug which precluded anaphylactic symptoms, no comparison of the latter was possible. Statistical comparison of the disappearance values for the three groups gave the following result: 30-minute group compared to 1-minute group $P < 0.001$; 30-minute group compared to 10-minute group, $0.001 < P < 0.01$; 10-minute group compared to 1-minute group, $0.01 < P < 0.05$.



Fig. 3. Reappearance of mast-cells in the nasal mucosa of guinea pigs at varying intervals after anaphylactic disappearance. Mast-cell population expressed in percent of the population on the untreated control side.

III Reappearance of mast cells following anaphylactic disappearance

Local application of antigen upon the nasal mucosa of sensitized guinea pigs produces a rapid and extensive disappearance of mast cells (Boréus 1960) The purpose of the present experiment was to determine the time required for reappearance of the cells. The above-described procedure for intranasal administration of antigen was employed, 5 portions of 2 mg antigen each being dissolved in 0 02 ml saline and introduced into the left nasal cavity These volumes were too small to be aspirated and thus lead to anaphylactic shock.

Following this treatment the animal had, on the right side, an intact nasal mucosa and on the left a mucosa which had undergone a local anaphylactic reaction with mast-cell depletion. The animals were killed after varying intervals and the difference in mast-cell count between the right and left sides was estimated. The results of these experiments are shown in Fig 3 The application of antigen resulted in a sharp decline in mast-cell population. Total disappearance of the mast cells was at no time observed, however After about 3 weeks the mast cells began to reappear They were smaller than normal and the metachromatic material was sparse and faintly stained. Within approximately 7 weeks, most of the mast cells had reappeared and had regained their usual size and staining characteristics.

Attempts were made to maintain the low mast-cell population following local anaphylaxis. In several animals, therefore, antigen was reapplied intranasally 5 weeks after the first treatment. When the animals were killed a few weeks later however the left nasal cavity and in some cases the right one was found to be severely infected and suppurative. The inferior nasal concha was entirely or partly necrotic and in some animals no concha whatsoever remained. Histological examination of the remnants of the mucosa revealed, in some instances, a tremendous accumulation of mast cells distributed throughout the concha.

Some of the animals were challenged with antigen following removal of the specimens. They all developed severe shock, showing that the intranasal administration of antigen had not been sufficient to produce appreciable desensitization (*cf* Boréus 1960 a)

Two animals treated 8 weeks earlier with intranasally applied antigen were challenged with the standard dose of antigen intra-arterially Before challenge, one specimen was taken from each side to make certain that the mast cells had returned in the usual way The antigen injection produced fatal shock in both animals, and gave rise to a strong mast-cell reaction 77.3 ± 3.2 %. This experiment indicates that the mast cells which have reappeared following anaphylactic reaction, disappear at approximately the former rate upon further antigen administration.

Discussion

The causal relationship between formation of antibodies and development of active anaphylaxis was early confirmed by demonstration of the passive anaphylactic state in which a normal animal receives antibody formed by another animal. An essential factor in passive anaphylaxis in the guinea pig is the interval between the two injections of antibody and antigen. This latent period might reflect the time required for the antibody to become fixed on or in the cells. Such a hypothesis is supported by the finding of BENACERRAF and KABAT (1949) and that of OVARY and BIER (1953) that the latent period can be reduced with increasing doses of sensitizing antiserum.

Though cellular fixation of antibody may take place more or less uniformly in the body it seems that, quantitatively only a few types of cells play a major role in anaphylaxis. In the guinea pig, the destruction of tissue mast cells appears to be a principal factor in the genesis of anaphylactic symptoms. Species differences, however are important. For instance, rabbit platelets, which contain serotonin as well as most of the blood histamine, are disintegrated in the blood by antigen *in vitro* (HUMPHREY and JAQUES 1955) and *in vivo* (WAALKES *et al.* 1957) It is probable that these cells figure predominantly in rabbit anaphylaxis. This may explain the early observation that no latent period is required for passive sensitization in this species, when both antibody and antigen are administered intravenously (DRAGSTEDT *et al.* 1940 DRAGSTEDT 1941 ROCHA E SILVA 1942) It is unlikely that platelets are of major importance in other species such as guinea pig rat and man, since these species do not produce the high levels of circulating antibodies demonstrable in rabbit (WAKSMAN 1959) Moreover it was shown by HUMPHREY (1955) that anaphylactic shock can be produced in guinea pigs completely deprived of circulating platelets by the use of platelet antiserum.

HUMPHREY and MOTA (1959) incubated mesentery from passively sensitized guinea pigs with antigen and demonstrated a diminution of mast-cell numbers. The present investigation shows heavy mast-cell depletion in intact tissue on antigen administration *in vivo* to a passively sensitized guinea pig The finding that the mast-cell reaction may be elicited on the sixth but is no longer elicitable on the eleventh day after injection of the antiserum is in accord with early

observations on the duration of the passive anaphylactic state (WEIL 1917 COCA and KOSAKAI 1920) The antibody-containing serum was not only responsible for the passive anaphylaxis but initiated, at the same time, active antibody production which was demonstrable 15 days later when the mast cells showed marked disintegration in response to injection of the rabbit serum.

Local anaphylaxis without general anaphylactic shock, but manifested as disappearance of tissue mast cells, was observed in the nasal mucosa on topic application of antigen solution thereto (BORÉUS 1960) The reaction was rapid and uniform throughout the nasal concha, indicating that the antigen was easily absorbed through that tissue. This is corroborated by the finding in this study that active sensitization of the whole animal is readily induced via the intranasal route and is demonstrable both as anaphylactic shock and as a mast cell reaction to antigen. STRÖMME (1952) reported that local application of rye and birch pollen to the nares of guinea pigs could produce active sensitization, manifested as a nasal discharge and as sneezing and scratching of the nose when the animals were challenged by pollen applied to the nares. Intravenous injection of pollen extract produced anaphylactic shock symptoms but caused no death. In this investigation an attempt was made to duplicate these experiments with the mast-cell method. Rye and birch pollen was given parenterally in large doses to normal guinea pigs. Challenge a few weeks later by means of intra-arterial or topic administration of pollen extracts, however gave rise to neither mast-cell reaction nor anaphylactic shock symptoms.

Much work has been devoted to inhibition of anaphylactic reactions. For this type of study *in vitro* methods such as measurement of histamine liberation from tissues, of mast-cell reaction, and of smooth-muscle contraction have been widely used since they permit numerous experiments on one animal. Studies *in vivo* have been based upon estimation of the intensity of anaphylactic shock symptoms. This intensity is difficult to evaluate quantitatively and subjective error is not easily avoided. *In an earlier paper (BORÉUS 1960 a)* inhibition of anaphylactic mast-cell reaction was studied quantitatively during desensitization processes *in vivo* The same method is here used to study the effects of ACTH and cortisone, of heating, and of slow antigen administration upon the mast-cell sensitivity to antigen

Despite extensive study of ACTH and cortisone, the pharmacodynamics of these hormones in allergy remain obscure. Their influence on anaphylactic reactions is controversial. Although neither dose-response nor time curves were plotted in this study the negative results with ACTH and cortisone respecting anaphylactic mast-cell response in actively sensitized guinea pig tend to substantiate the view held by most authors engaged in guinea pig studies with various techniques (LEGER LEITH and ROSE 1948, FRIEDLAENDER and FRIEDLAENDER 1950 MALKIEL 1951) In the present experiments the dosages of cortisone and of ACTH (which has no direct action of its own) were fairly high, and any definite inhibitory action of those drugs would have lowered the disap-

pearance values. The dose of antigen was the same as that used earlier in studies of inhibition resulting from desensitization (Boréus 1960 a)

The effect of the adrenocortical hormones upon formation of anaphylactic antibody is likewise unclear. Most studies of this problem seem to have been done in rabbits (for references, see Rose 1959). That ACTH and cortisone may depress antibody levels in this species is generally agreed. Germuth, Ottinger and Oyama (1952) reported similar suppression in the guinea pig. In the present experiment high doses of both hormones failed to depress either mast-cell reaction to antigen or the anaphylactic shock. It is possible that even a meager formation of antibody suffices to sensitize the tissue mast cells (and probably other cells as well), whereby a full anaphylactic response may still occur.

Asboe Hansen (1952) reported that during administration of cortisone to humans, rabbits, mice and guinea pigs, the number of mast cells in the connective tissue diminishes and vacuolization as well as alteration in the shape and outlines of mast cells take place. Similar effects were noted in the hamster by Fulton and Maynard (1953) and in the rat by Cavallero and Braccini (1951). On the other hand, Devitt, Pirozynski and Samuels (1953) found that cortisone produced no change in mast-cell frequency in various rat tissues in experiments involving numerical cell counts. ACTH and cortisone moreover had no effect on the total number of mast cells in rat tissues (Smith and Lewis 1954) or in the cheek pouch of hamsters (Smith and Lewis 1955). The present results accord with these findings. No decrease in mast-cell frequency was detected in the nasal mucosa of guinea pigs during treatment with ACTH and cortisone, nor was a specific change in the appearance of the cells observed.

The thermal inhibition of anaphylaxis has been adduced by several authors as evidence of an enzymatic stage in this reaction. *In vivo* experiments on guinea pigs were performed by Mongar and Schild (1956) who noticed that anaphylactic shock was inhibited in animals which had been heated before challenge. The present study suggests that this phenomenon may stem from inhibition of the mast-cell reaction to antigen.

Prolongation of the injection time diminished the mast-cell response, although the 30-minute values showed a high degree of variation. A possibl explanation is that the tissue antibodies were partly saturated with antigen and the cells thus desensitized. This kind of anaphylactic inhibition has long been recognized (Friedberger and Mita 1911, Lewis 1919).

Sensitized guinea pigs treated by pplication of antigen to the nasal mucosa exhibited almost total mast-cell depletion of the mucosa on the treated side but normal mucosa on the untreated side. The procedure may be utilized for study of mucosal mast-cell function. In this investigation the rate of reappearance of mast cells following anaphylaxis was investigated. It appears that such study on the guinea pig has been previously reported. The incr

mast-cell population was found to be rather slow and of the same order as the recovery of histamine in the skin of dog and rat following injection of compound 48/80 (FELDBERG and TALESNIK 1953). It was also found comparable to the restoration rate of histamine and mast cells in the subcutaneous connective tissue and the ear skin of the rat after injection of the same drug (RILEY and WEST 1955). The reappearing mast cells were found to react promptly to readministered antigen. New formed cells thus adopt the induced anaphylactic sensitivity pattern of the individual.

References

ASBOE-HANSEN, G. The mast cell. Cortisone action on connective tissue. *Proc. Soc. exp. Biol.* (N. Y.) 1952. *80*. 677—679.

BENACERRAF, B. and E. A. KABAT. A quantitative study of passive anaphylaxis in the guinea pig. V. The latent period in passive anaphylaxis in its relation to the dose of rabbit anti-ovalbumin. *J. Immunol.* 1949. *62*. 517—522.

BORÉUS, L. O. Quantitative studies on the anaphylactic mast-cell reaction in vivo in the guinea pig. *Acta physiol. scand.* 1960. *48*. 431—438.

BORÉUS, L. O., A study of the anaphylactic mast-cell reaction in vivo following desensitization of sensitized guinea pigs. *Acta physiol. scand.* 1960 a. *50*. 375—384.

CAVALLERO, C. and C. BRACCINI. Effect of cortisone on the mast cells of the rat. *Proc. Soc. exp. Biol.* (N. Y.) 1951. *78*. 141—143.

COCA, A. F. and M. KOSAKAI, Studies in anaphylaxis. *J. Immunol.* 1920. *5*. 297—319.

DEVITT, J. E., W. J. PIROZYNSKI and P. B. SAMUELS, Mast cell resistance to hormonal influence. *Proc. Soc. exp. Biol.* (N. Y.) 1953. *83*. 335—337.

DRAGSTEDT, C. A., Anaphylaxis. Physiol. Rev. 1941. *21*. 563—587.

DRAGSTEDT, C. A., M. R. DE ARELLANO, A. H. LAWTON and G. P. YOUMANS, Passive sensitization of rabbits blood. *J. Immunol.* 1940. *39*. 537—542.

FELDBERG, W. and J. TALESNIK, Reduction of tissue histamine by compound 48/80. *J. Physiol.* (Lond.) 1953. *120*. 550—568.

FRIEDBERGER, E. und S. MITA, Ueber eine Methode, grössere Mengen artfremden Serums bei überempfindlichen Individuen zu injizieren. *Dtsch. med. Wschr.* 1912. *38*. 204—207.

FRIEDLAENDER, S. and A. S. FRIEDLAENDER, The effect of pituitary adrenocorticotropic hormone (ACTH) on histamine intoxication and anaphylaxis in the guinea pig. *J. Allergy* 1950. *21*. 303—309.

FULTON, G. P. and F. L. MAYNARD. Effect of cortisone on tissue mast cells in the hamster cheek pouch. *Proc. Soc. exp. Biol.* (N. Y.) 1953. *84*. 259—260.

GERMUTH, F. G. Jr., B. OTTINGER and J. OYAMA, Influence of cortisone on experimental hypersensitivity and circulating antibody in the guinea pig. *Proc. Soc. exp. Biol.* (N. Y.) 1952. *80*. 188—191.

HUMPHREY, J. H. and R. JAQUES, The release of histamine and 5-hydroxytryptamine (serotonin) from platelets by antigen-antibody reactions (in vitro). *J. Physiol.* (Lond.) 1955. *128*. 9—27.

HUMPHREY, J. H. and I. MOTA, The mechanism of anaphylaxis: Specificity of antigen-induced mast cell damage in anaphylaxis in the guinea pig. *Immunology* 1959. *2*. 31—43.

LEGER, J., W. LEITH and B. ROSE. Effect of adrenocorticotropic hormone on anaphylaxis in the guinea pig. *Proc. Soc. exp. Biol.* (N. Y.) 1948. *69*. 465—467.

LEWIS, J. H., Slow intravenous injection of antiserum to prevent acute anaphylactic shock. *J. Amer. med. Ass.* 1919. 329—330.

MALKIEL, S., The influence of ACTH and cortisone on histamine and anaphylactic shock in the guinea pig. *J. Immunol.* 1951. *66*. 379—384.

MONGAR, J. L. and H. O. SCHILD, Inhibition of the anaphylactic reaction in vitro and in vivo. *J. Physiol.* (Lond.) 1956. *132.* 50 P

OVARY, Z. and O. G. BIER, Quantitative studies on passive cutaneous anaphylaxis in the guinea pig and its relationship to the Arthus phenomenon. *J. Immunol.* 1953. *71.* 6—11.

RILEY, J. F. and G. B. WEST, Histamine-liberation in the rat and mouse. *Arch. int. Pharmacodyn.* 1955. *102.* 304—312.

ROCHA E SILVA, M., The histamine theory of anaphylactic shock, with special reference to anaphylaxis in the rabbit. *A. M. A. Arch. Path.* 1942. 33. 387—408.

ROSE, B., Hormones and allergic responses. In: Mechanisms of hypersensitivity. Int. symp. sponsored by the Henry Ford Hospital. *Little, Brown and Co.* Boston 1959. pp. 600.

SMITH, D. E. and Y. S. LEWIS, Influence of hypophysis and adrenal cortex upon tissue mast cell of the rat. *Proc. Soc. exp. Biol.* (N. Y.) 1954. *87.* 515—518.

SMITH, D. E. and Y. S. LEWIS, Influence of hypophysis and of adrenal cortex upon tissue mast cell of the hamster. *Proc. Soc. exp. Biol.* (N. Y.) 1955. *88.* 631—634.

STRÖMER, O., Studies on the histopathology of pollinosis. *Acta oto-laryng.* (Stockh.) 1952. Suppl. 104.

WAALKES, T. P., H. WEISSBACH, J. BOZICEVICH and S. UDENFRIEND, Serotonin and histamine release during anaphylaxis in the rabbit. *J. Allergy* 1957. *36.* 1115—1120.

WAKSMAN, B. H., The toxic effects of the antigen-antibody reaction on the cells of hypersensitive reactors. In: Cellular and humoral aspects of the hypersensitive states. Symp. held at the N. Y. Acad. Sci. Ed. by H. S. Lawrence. *Hoeber and Harper.* New York 1959. pp. 160.

WEIL, R., Studies in anaphylaxis. XX. The reciprocal relations of antigen and antibody within the cell. A contribution to cellular immunology. *J. Immunol.* 1917. *2.* 469—499

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Quantitative Differences Between Guinea Pig Ileum and Uterus in the Schultz Dale Reaction

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Abstract

BORÉUS, L. O *Quantitative differences between guinea-pig ileum and uterus in the Schultz Dale reaction.* Acta physiol. scand. 1961 52 320—327 — Although the anaphylactic contraction of smooth muscle *in vitro* (the SCHULTZ DALE reaction) is of the same type, intensity and duration in guinea pig ileum and uterus, some quantitative differences between these two organs are demonstrable. To produce equivalent contractions by addition of histamine to the bath, ten times higher dose was required for uterus than for ileum. In the case of uterus, this amount was more than three times the total histamine content. Uterus released much greater amounts of smooth-muscle stimulating substance during anaphylactic contraction than did ileum. In terms of histamine, 9 and less than 0 4 %, respectively of the total content was released. Moreover the mast-cell disappearance, demonstrable only with the highest antigen doses used, was more pronounced in uterus.

A characteristic feature of anaphylaxis in the guinea pig is smooth-muscle spasm. The pronounced contraction of the bronchiolar muscles, as well as the discharge of urine and faeces, are well-known phenomena in the anaphylactic shock syndrome. SCHULTZ (1910) and DALE (1913) demonstrated the antigen-induced contraction *in vitro* of the isolated ileum or uterus (the "SCHULTZ DALE reaction") Similar contraction of isolated guinea pig tissues has been demonstrated in strips of bronchus and pulmonary artery (GROVE 1932)

The discovery of histamine in the mast cells (RILEY and WEST 1953) has given possibilities of new experimental approaches to anaphylactic phenomena Incubation experiments with sensitized guinea-pig tissues (BORÉUS and

CHAKRAVARTY 1960) showed that anaphylactic disappearance of mast cells was correlated to the appearance of both histamine and another smooth-muscle stimulating principle, the slow reacting substance (SRS)

In the experiments reported here the mast-cell reactions occurring in isolated ileum and uterus of sensitized guinea pigs following the SCHULTZ DALE reaction were compared By means of a non-sensitized strip of guinea-pig ileum suspended in the same bath, the amount of released smooth-muscle stimulating substance was measured simultaneously

Methods

Female virginal guinea pigs, weighing about 300 g were sensitised with one subcutaneous and, 3 days later one intraperitoneal injection of 100 mg crystalline egg albumin. After an interval of 3—5 weeks the animals were sacrificed by blow on the head and the ileum or uterus was removed, washed and immersed in Tyrode solution at room temperature pending use.

Segments of ileum or uterus, about 1.5 cm long, were suspended in 5 ml aerated bath at 37° C. The fluid was Tyrode solution containing glucose (1 mg/ml) The bath was washed by overflow The suspended segment, called the SCHULTZ DALE segment (SD segment) was attached to a frontal lever and its contractions were recorded on the kymograph drum. An adjacent strip of ileum or uterus, called the control segment, was immersed in Tyrode solution t 37° C during the experiment. The olumes of added drugs (histamine dihydrochloride or egg albumin, both dissolved in Tyrode solution) were 0.2 ml or less.

In the indicator segment experiments, designed to reveal any release of smooth-muscle stimulating agents into the bath from the SD segment during contraction, a strip of ileum from non-sensitized guinea pig was suspended in the bath together with the SD segment. Its contractions were recorded by another lever A similar technique has been used by DWORETZKY (1959)

The experimental procedure was as follows When both SD strip and indicator strip had relaxed and stable base lines had been secured, varying doses of histamine were added to the bath in order to obtain dose-response curves. Antigen to final concentration of 0.004 mg/ml was then added and allowed to remain in the bath for 3-minut period. Any contraction of the SD strip and indicator strip during this period was estimated in terms of histamine by comparative reference to the dose-response curve for the latter drug. At the end of the 3-minute period the antigen was washed out of the bath. When the SD strip had returned to the initial base-line, it was removed and weighed. A piece of the strip was cut off and placed in the fixative for subsequent mast-cell count. The rest of the strip was weighed, homogenized, and boiled in HCl. After neutralization, the extract was assayed for histamine on atropinized (1.5×10^{-6} M tropine sulphate) guinea-pig ileum. A small piece from the control strip was fixed for histologic examination; the remainder was weighed and homogenized for histamine assay All histamine values are given as the base.

The histologic treatment comprised fixation, sectioning and staining as described earlier (BOSTRÖM 1960) For each strip, the total number of recognizable mast cells in four different sections was estimated and expressed as number of cells per square unit of section, the area of which was estimated by means of square net inserted in the ocular The mast-cell count of the SD segment was compared with that of the control segment and any depletion of the cell population, e., "mast-cell disappearance" was expressed as percentual decrease from the control segment. (A negative value thus denotes greater population of mast cells in the SD segment than in the control segment.)

By means of this procedure the following values could be obtained (1) The degree of SD contraction in terms of histamine (2) the amount, in terms of histamine, of smooth-muscle contracting substance liberated, over a 3-minute period, from the SD strip into the bath during anaphylactic contraction (3) the histamine content of the SD strip and of the control strip after the experiment and (4) the mast-cell disappearance value for the SD strip

Results

Distribution of mast cells in guinea-pig ileum and uterus. Most of the mast cells in guinea pig ileum are located in the basal part of the mucosa, in the submucosal layer and, to a somewhat lesser degree, in the circular part of the muscular coat. In counts of 20 different strips, the mast-cell population was 3.2 times higher in the submucosa and the basal third of the mucosa than in the circular muscle layer

Uterine mucous membrane is poor in mast cells. Only in the deepest layer was some mast-cell accumulation observed. The muscularis mucosae and the submucosa were comparatively rich in mast cells and in a series of counts in 8 different strips showed a frequency 2.9 times higher than that of the outer muscular layer

Several preliminary experiments with "differential" counts had disclosed that the mast cells in different layers of ileum and uterus reacted similarly and in like degree to antigen. In these experiments, therefore, the total numbers of mast cells in whole sections were used for the comparisons.

Experiments with indicator strips in the bath. The principal results are detailed in Table I and will be reviewed column by column.

(1) The standard antigen concentration of 0.004 mg/ml invariably produced substantial SCHULTZ DALE contraction in both ileum and uterus, and of like type, intensity and duration. The contraction started after a latency of 10—30 seconds. The maximum was reached after 15—60 seconds, when slow relaxation occurred. The initial base line was usually reached 10—20 minutes later It will be seen that the amount of histamine needed to duplicate the reaction was much higher for uterus than for ileum, a finding which is consistent with the well-known difference in the sensitivity of these two tissues to histamine. On occasion the insensitivity of uterus to histamine was so great that the standardization had to be done *after* the anaphylactic reaction in order to avoid too high a concentration of histamine in the bath and possible reduction of the subsequent sensitivity to histamine during the SCHULTZ DALE reaction, as described by SCHILD (1936) It should be noted that the amount of histamine needed for duplication of the SCHULTZ DALE contraction in uterus is more than three times the total amount of histamine in the strip

It was observed that storing of the strips in Tyrode solution at room temperature gradually decreased their ability to contract upon antigen administration but did not produce a commensurable loss of sensitivity to histamine.

Table I Results of experiments with Schultz-Dale contraction of ileum and uterus in the presence of indicator strip of ileum in the same bath. Final antigen concentration 0 004 mg/ml. Exposure time 3 minutes

Tissue	Number of experiments	1	2		3		4
		Schultz-Dale contraction (in terms of μg histamine)	Liberated contracting substance (in terms of histamine)		Histamine content after experiment		Mast-cell disappearance %
			μg/strip	% of histamine content of the strip	SD strip μg/g	Control strip μg/g	
Ileum	9	0.16±0.05	<0.007 ± 0.008	<0.4 ± 0.2	10.2±1.4	10.1±1 4	−7.8 ± 6.6
Uterus	12	1 78±0.39	0.032 ± 0.006	9.3 ± 2.5	7.2±1 4	7.3±1.2	13.0 ± 8.6

(2) Another striking difference between ileum and uterus is evident from the values for the contracting substance released into the bath. This amount, in terms of histamine, was about 5 times higher in the uterus than in ileum. In 5 of the 9 experiments with sensitized ileum, no contraction at all was observed in the indicator strip. In these 5 instances the lower limit for histamine sensitivity was 0.0012 or 0.0018 μg histamine. These limit values were taken as maximal values for liberated contracting substance and are included in the mean values for ileum in column 2 of Table I. This means that the true values for ileum are even lower than 0 007 μg and 0.4 % respectively

The contraction of the indicator segment began a few seconds after the onset of the SCHULTZ DALE contraction and rose slowly to its maximum. When, at the end of the 3-minute period, the bath was washed, the indicator strip immediately relaxed, although the SD strip of ileum or uterus was still in contraction.

(3) Following contraction the total histamine content in the SD strips was not significantly lower than that in the control strips. Assuming, however that the liberated contracting substance was in fact histamine, a quantity so small would not be demonstrable by comparison of the histamine content of the strips, since the variation of these values is too wide.

(4) It is evident that the variation of the mast-cell disappearance values is wide. Although neither value alone significantly differs from zero the two values differ from each other (P = 0 01 – 0 05)

A definite difference between the small intestine and the uterus in respect to mast-cell reactivity as well as to histamine liberation on addition of antigen *in vitro* was discovered earlier (BORÉUS and CHAKRAVARTY 1960) when the antigen concentration was 1 mg/ml and the incubation time 10 min. Studies of the mast-cell disappearance during the SCHULTZ DALE reaction were therefore extended.

Table II. Percentual mast-cell disappearance in ileum and uterus of sensitized and non-sensitized guinea pigs following Schultz Dale contraction. Exposure time 30 minutes. P values from statistical comparison of sensitized and non-sensitized animals

	Sensitized		Non-sensitized
	Final antigen concentration		
	0.4 mg/ml	4 mg/ml	
Ileum	10.6 ± 4.7	16.0 ± 6.2	− 2.3 ± 2.8
	n = 7	n = 4	n = 8
	P 0.05 – 0.01	P 0.05 – 0.01	
Uterus	25.5 ± 7.7	34.0 ± 4.7	− 1.6 ± 5.6
	n = 6	n = 6	n = 8
	P 0.05 – 0.01	P < 0.001	

In this new series of experiments (without indicator strips) anaphylactic contractions were produced with antigen in final concentrations of 0.4 and 4 mg/ml during a 30-minute period. In order to obviate any possible effect of the muscle contraction itself upon the mast-cell population a series of ileal and uterine strips from non-sensitized animals were suspended in the bath an adjacent strip serving as a control in the usual way. After maximal relaxation of the strip, antigen (which was shown to have no effect at all on the non-sensitized strip) as well as a suitable dose of histamine were added in order to produce contraction of the strip. The histamine was left in the bath for a sufficient length of time to reproduce the intensity and duration of a SCHULTZ-DALE contraction. (In several experiments additional doses of histamine had to be administered, since histamine contraction is usually of shorter duration than anaphylactic contraction.) The results of all these experiments are summarized in Table II. Mast-cell depletion following the SCHULTZ DALE reaction was found in both ileum and uterus, although it was comparatively slight. The values were higher with the higher dose of antigen. The uterus showed a stronger mast-cell reaction than the ileum, the difference being most marked at the higher dose. The histamine-produced contractions of the non-sensitized strips gave no mast-cell reaction.

Discussion

SCHILD (1939) showed that different guinea-pig tissues, when incubated with antigen, released different amounts of histamine. Uterus for instance, released about 20 times as much histamine as ileum, which was very resistant to antigen. A similar discrepancy between uterus and jejunum was found by BORÉUS and CHAKRAVARTY (1960). They pointed out that incubation of jejunum was

followed by the appearance of very small amounts of histamine, no slow reacting substance (SRS, a lipid soluble smooth muscle stimulating principle) and no mast-cell disappearance although the content of both histamine and mast cells was high. Incubation of uterus, on the other hand, resulted in anaphylactic appearance of histamine and SRS and in disappearance of mast cells.

A similar difference is now demonstrated during the SCHULTZ DALE reaction despite the apparent similarity in onset, intensity and duration of the contraction. Thus, much higher amounts of smooth-muscle contracting substance was liberated from uterus than from ileum. Moreover with higher antigen doses the observed mast-cell disappearance was more conspicuous in uterus than in ileum.

Is liberation of contracting agents from tissue cells essential to the anaphylactic contraction? Assuming that such liberation is the direct cause (and not the result) of the contraction and that a part at least of the liberated substance is histamine, it may be asserted on the basis of the results, that the small amount released in ileum is compensated by the high sensitivity of the muscle cell to this drug, and that the greater release in uterus corresponds to a lesser sensitivity

This investigation was not designed to study the nature of the released agents. The results cannot settle the question whether histamine is of major importance in the SCHULTZ DALE reaction, since neither pharmacologically induced duplication of the anaphylactic contraction nor quantitative determination of released smooth-muscle stimulating principles can help to establish the precise concentration of these agents at receptor sites within the tissue. Several authors, however have called attention to numerous discrepancies in the histamine theory of the SCHULTZ DALE reaction (SCHILD 1936, 1939 FELDBERG 1941 MAYER 1950 HAWKINS and ROSA 1956 REUSE 1956 DWORETZKY 1959) It must be pointed out that the arguments for or against the role of histamine in the reaction are based upon the assumption that the receptors involved are the same whether histamine is liberated in the tissue or whether it is added to the bath solution.

Other agents which have been ascribed a role in the SCHULTZ DALE reaction are acetylcholin and serotonin (NAKAMURA 1941 DANIELOPOLU et al. 1948, GEIGER, HILL and THOMPSON 1956) On the basis of experiments with various inhibitors, GEIGER and ALPERS (1959) concluded that at least three humoral agents, histamine, acetylcholine and serotonin, are involved in the reaction. Other investigators, however have not succeeded in demonstrating the release of serotonin in the anaphylactic contraction of guinea-pig smooth muscle (FINK and GARDNER 1958 BORÉUS and WESTERHOLM 1961) Though SRS could not be detected after incubation of the small intestine with antigen, on incubation of uterus it occurred in amounts closely corresponding to those of histamine (BORÉUS and CHAKRAVARTY 1960) Quantitative evaluation of the role of SRS and of other agents is an intricate problem since it must be assumed

they may interact. For instance, BROCKLEHURST (1956) as well as CHAKRAVARTY HÖGBERG and UVNÄS (1959) showed that SRS sensitized isolated guinea-pig ileum to histamine.

It is possible that the anaphylactic contraction is the result of a combination of antigen and antibody in or on the muscle cell itself. Further elucidation of the SCHULTZ DALE reaction will require more study of the response to antigen of the smooth-muscle cell itself. LEHOTAN and HAŠÍK (1959) have shown that, on addition of antigen, actomyosin fibers from smooth muscle of sensitized guinea pigs exhibit contraction analogous to that associated with the SCHULTZ DALE reaction. Their experiments on this "nerve free model of muscle" indicate that liberation of contracting substances from other cells (such as mast cells) may not be essential to the anaphylactic contraction of the smooth muscle cell.

References

BORÉUS, L. O. Quantitative studies on the anaphylactic mast-cell reaction in vivo in the guinea pig. *Acta physiol. scand.* 1960. *48.* 431—438.

BORÉUS, L. O. and N. CHAKRAVARTY. Tissue mast cells, histamine and "slow reacting substance" in anaphylactic reaction in guinea pig. *Acta physiol. scand.* 1960. *48.* 315—322.

BORÉUS, L. O. and B. WESTERHOLM, To be published.

BROCKLEHURST, W. E., A slow reacting substance in anaphylaxis — "SRS-A". Ciba Foundation symposium on histamine. *J. & A. Churchill Ltd.* London 1956. pp. 175—179.

CHAKRAVARTY, N., B. HÖGBERG and B. UVNÄS, Mechanism of the release by compound 48/80 of histamine and of a lipid-soluble smooth muscle stimulating principle ("SRS"). *Acta physiol. scand.* 1959. *45.* 255—270.

DALE, H. H. The anaphylactic reaction of plain muscle in the guinea pig. *J. Pharmacol.* 1913. *4.* 167—223.

DANIELOPOLU, D., A. RUDEANU, I. BRUCKNER and S. SIMONESCU, Libération d'acétylcholine dans le choc paraphylactique (anaphylactique). *C. R. Soc. Biol.* (Paris) 1948. *142.* 737—738.

DWORETZKY, M. Role of histamine or other released substances in anaphylaxis in isolated guinea pig ileum. *Amer. J. Physiol.* 1959. *197.* 51—53.

FELDBERG, W. Histamine and anaphylaxis. *Ann. Rev. Physiol.* 1941. *3.* 671—694.

FINK, M. A. and C. E. GARDNER, Anaphylaxis in guinea pig. Improbability of release of serotonin in the Schultz-Dale reaction. *Proc. Soc. exp. Biol.* (N. Y.) 1958. *97.* 554—557.

GEIGER, W. B. and H. S. ALPERS, The mechanism of the Schultz Dale reaction. *J. Allergy* 1959. *30.* 316—328.

GEIGER, W. B., E. M. HILL and M. THOMPSON, A study of the mechanism of the Schultz-Dale reaction. *Proc. Soc. exp. Biol.* (N. Y.) 1956. *92.* 793—796.

GROVE, E. F. Studies in anaphylaxis. IV. On the Simond explanation of the different pathology of acute anaphylactic shock in different animal species. On the rôle of unstripped muscle in acute anaphylaxis in the rabbit. *J. Immunol.* 1932. *23.* 147—15.

HAWKINS, D. F. and L. M. ROSA, Some discrepancies in the histamine theory of anaphylaxis in smooth muscle. Ciba Foundation symposium on histamine. *J. & A. Churchill Ltd.* London 1956. pp. 180—182.

LEHOTAN, O. and A. HAŠÍK, Anafylaktická kontrakcia aktomyozínového vlákna (Anaphylactic contraction of actomyosin fibre). Bratislavské lekárske Listy 1959. *39.* 600—610.

MAYER, R. L., The activity of Pyribenzamine and related compounds with special reference to their mode of action. *Ann. N. Y. Acad. Sci.* 1950. *50.* 1127—1141.

NAKAMURA, K., Nouvelle opinion sur la production du choc anaphylactique, spécialement sur le rôle de l'acétylcholine, dans l'anaphylaxie. *Jap. J. exp. Med.* 1941. *19.* 31—42.
REUSE, J. J. *Antihistamine drugs and histamine release, especially in anaphylaxis.* Ciba Foundation symposium on histamine. *J. & A. Churchill Ltd.* London 1956. pp. 150—154.
RILEY, J. F. and G. B. WEST. The presence of histamine in tissue mast cells. *J. Physiol.* (Lond.) 1953. *120.* 528—537.
SCHILD, H. O., The reaction of the guinea-pig' uterus immersed in histamine solution to histamine and anaphylaxis. *J. Physiol.* (Lond.) 1936. *86.* 51 P—53 P.
SCHILD, H. O. Histamine release in anaphylactic shock from various tissues of the guinea pig. *J. Physiol.* (Lond.) 1939. *95.* 393—403.
SCHULTZ, W. H., Physiological studies in anaphylaxis. I. The reaction of smooth muscle of the guinea-pig sensitized with horse serum. *J. Pharmacol.* 1910. *1.* 549—567.

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Studies on the Peripheral Circulation and Metabolism in Man

I. Oxygen utilization and lactate-pyruvate formation in the legs at rest and during exercise in healthy subjects

By

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Abstract

CARLSON, L. A. and B. PERNOW. *Studies on the peripheral circulation and metabolism in man. I. Oxygen utilization and lactate-pyruvate formation in the legs at rest and during exercise in healthy subjects.* Acta physiol. scand. *1961. 52. 328—342.* — Arterial and femoral venous oxygen and lactate-pyruvate was studied at rest, during and after leg exercise in 15 healthy human males. At rest in the supine position the femoral venous oxygen saturation was 53—81 per cent. A few minutes after starting work, the venous oxygen saturation was 22—42 per cent and a slight but steady decrease was observed on continued work. Five minutes after work, the venous oxygen saturation was significantly higher than at rest before work. *The pH of the femoral venous blood decreased* from mean 7.41 to 7.23 at maximal load. The venous lactate rose during exercise to 4.45—8.66 mM/liter and the venous-arterial lactate difference to mean 0.85 mM/liter at a heart rate of *160—187/min* te. After exercise the lactate decreased successively in most cases but had even 30 min later not reached the initial level before work. The venous pyruvate concentration was 0.09—0.15 mM/liter at rest, and increased successively during exercise to 0.22—0.35 mM/liter at the highest loads. A further increase in pyruvate was obtained during the first minutes after work. The increase in excess of lactate as calculated from the lactate-pyruvate ratio, during exercise was practically parallel to that in total lactate indicating that during work the total lactate is a good indicator of the adequacy of oxygen supply to the

tissues. After exercise, however there was discrep ncy between the total and excess of lactate, the latter decreasing much more rapidly No difference was obtained between older and younger subjects concerning increase in pulse rate or changes in oxygen saturation and blood lactate-pyruvate during exercise. After work lactate decreased more slowly in the older subjects than in the younger

Studies on the metabolism of resting and exercising tissues are greatly facilitated by the supply of arterial as well as venous blood draining the tissue, which permits analyses of arteriovenous (AV) differences of the different metabolites. In man this technique has been successfully used in studies of the metabolism of heart muscle *in vivo* by BING and co-workers (for references, see BING *et al* 1954) However for many reasons it has not been applied to the same extent in studies on the metabolism of peripheral muscles. DONALD *et al* (1957) studied the femoral venous oxygen saturation and AV oxygen difference at rest and during supine leg exercise. The AV oxygen difference was found to increase almost linearly with increasing work, and to be closely related to the cardiac output. The calculated blood flow through the exercising leg increased about ten-fold with heavy work. Simultaneously the blood flow through resting tissue was markedly reduced (BISHOP *et al.* 1957) In a previous study (CARLSON nd PERNOW 1958) we were able to confirm the results of DONALD *et al.* (1957) The venous oxygen saturation was, at the beginning of light exercise, reduced to between one-half and one-third of that at rest in the supine position, whereas continuous work caused only a slight further decrease. During exercise performed with one leg only the venous oxygen saturation decreased at a similar rate n the resting leg as n the exercising one.

The increase in blood lactate concentration has been widely used as an index of the degree f anaerobic metabolism in exercising tissue (for references see HOLMGREN and STRÖM, 1959) During exhaustive work, a ten fold increase in blood lactate has been repeatedly observed (ASMUSSEN 1950 ÅSTRAND 1952) and in acute or chronic hypoxia either experimentally induced or in congenital heart disease the blood lactate has been found to increase t a lower working intensity than otherwise (DILL *et al* 1931 LUNDIN nd STRÖM 1947) Determination of the AV lactate difference gives the most reliable information about the adequacy of the oxygen pply to the working muscles. This was evident from a preliminary study in which the AV oxygen and lactate differences n exercising legs of patients w th impaired peripheral circulation were compared to corresponding values in healthy subjects (CARLSON and PERNOW 1959)

An accumulation of blood lactat s, however observed not only in muscular work, but also in conditions where hypoxia is not present, for example after hyperventilation and dmi istration of glucose, epinephrin or pyruvate (for references see HUCKABEE 1958 a) Consequently lactate alone is not a definite index of the adequacy of cellular oxygenation In all the aforementioned conditions (except hypoxia) in which lactate accumulates, a simulta eous

increase takes place in pyruvate (HUCKABEE 1958 a) With an inadequate oxygen supply reoxidation of the reduced diphosphopyridine nucleotide (DPNH) to its oxidized form (DPN) — necessary for the carbohydrate breakdown to form high energy phosphate bonds — is channelled by reduction of pyruvate to lactate. Hypoxia therefore results in an increased formation of lactate from pyruvate (ASMUSSEN 1950) As a measure of the degree of oxygen deficiency HUCKABEE (1958 a) used the calculation of "excess of lactate (XL) i. e. the lactate change exceeding that which is directly proportional to pyruvate changes.

The results obtained with the peripheral catheterization technique will be described in a series of papers. Our main purpose has been to study the degree of oxygen utilization and the lactate pyruvate formation in the legs during exercise in the healthy state, as well as in conditions characterized by a limitation of the working capacity It was hoped that this technique would provide a measure of the degree of aerobic and anaerobic work in the muscular tissue. The present paper comprises data from healthy subjects, 10 young and 5 older men, and forms the basis for comparison in the pathologic states presented in subsequent papers (CARLSON and PERNOW to be published)

Case Material

The case material consisted of two groups of healthy male volunteers, 10 aged 20—37 years, and 5 aged 48—56 years. All were clinically examined for cardiovascular and pulmonary function and found to be healthy

Methods

The investigation was carried out on fasting subjects in the morning. The brachial artery and femoral vein were punctured percutaneously and polyethylene catheters inserted by a technique described earlier (BERNEUS et al. 1954) The femoral vein was punctured about 2 cm below the inguinal ligament, and the catheter inserted about 8 cm distally Some minor hematomas were the only complication.

Exercise was performed in a sitting position on a bicycle ergometer and consisted of an uninterrupted series of work loads, starting with 150—300 kpm/minute and increasing every 5 min by 150—300 kpm/minute The work was performed with one leg only the pedal being fitted with a spring, to make its return passive. The one-leg exercise was chosen, since the subjects were also used as controls in another series of experiments, in which the same study was made in patients with unilateral circulatory disturbances of leg (CARLSON and PERNOW to be published) The heart rate was calculated from the ECG

The room temperature during the investigations was 20—23° C, and no arrangement was made to keep it constant.

Arterial and venous blood samples were drawn simultaneously at rest, as well as once at each work load during exercise and several times within 30 min of ending work. The blood was drawn into 20-ml glass syringes, their dead space being filled with one per cent heparin solution. All resting values refer to the supine position, and those obtained during exercise to the sitting position.

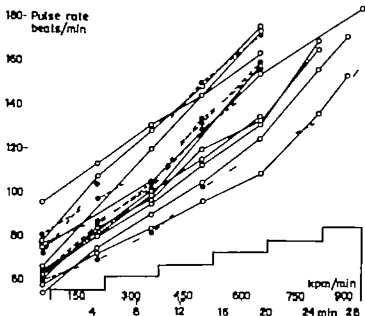


Fig. 1. Pulse rate at rest and at different loads during one-leg exercise with successively increasing load. ○———○ = young men; ● — — — — ● = older men.

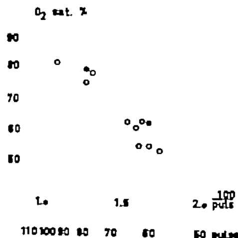


Fig. 2. Femoral venous oxygen saturation (%) at rest correlated to pulse rate (beats/min) at rest ○ = young men; ● = older men. Venous O saturation $= -37.1 \frac{100}{\text{pulse}} + 124.4$ ($p < 0.001$)

Duplicate estimates of the oxygen saturation and hemoglobin concentration were made spectrophotometrically the former by a slight modification of the method described by DRABKIN (1950). The oxygen content was calculated from the oxygen saturation and the oxygen capacity of the blood sample. All these determinations were made on each blood sample. The error of the methods as used in this laboratory has recently been reported (HOLMGREN and PERNOW 1959).

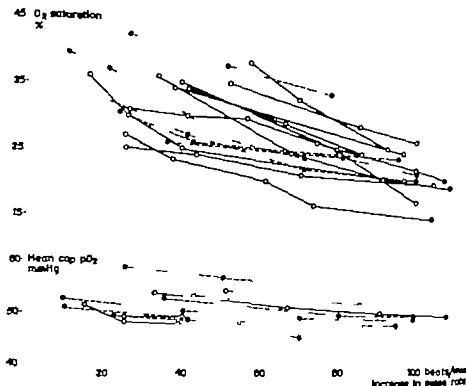


Fig. 3. Femoral venous oxygen saturation in % (upper diagram) and calculated mean capillary pO_2 in mm Hg (lower diagram) during one-leg exercise, correlated to rise in pulse rate from the resting value. Symbols as in Fig. 1

The pH of whole blood at 37° C was measured by glass electrode and potentiometric pH meter using phosphate buffer as standard. The standard error of a single determination was 0.002 pH units. Blood lactic acid was determined with Strom's (1949) modification of the colorimetric method of Barker and Summerson (1941). Pyruvate was determined according to the method of Friedemann and Haugen (1943) as modified by Huckabee (1956). The determinations were always made in duplicate. The precipitation of the blood by trichloracetic acid for lactate and pyruvate determinations was performed within 15 sec of withdrawing the blood. In preliminary experiments, the resting value for blood pyruvate obtained with this method was compared with that given by the paper chromatography method of Seligson and Shapiro (1952). Exactly the same values were obtained with the two methods. The error of the method (standard error of a single determination) calculated from duplicate determinations, was 0.013 mM/liter for lactate, and 0.005 mM/liter for pyruvate.

Results

Heart rate in relation to work loads

In every case, a linear relation existed between the increase in heart rate and the work load (Fig. 1). The pulse rate at rest, at the different work loads and after work, did not differ significantly in the older subjects from that in the younger ones.

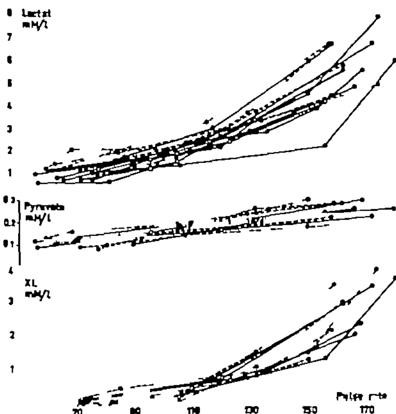


Fig. 4. Arterial lactate, pyruvate and "excess" lactate (XL) in mM/liter at rest and during exercise, correlated to relative work intensity (pulse rate during work). Symbols as in Fig. 1.

Oxygen saturation of arterial and venous blood

The oxygen saturation of arterial blood was in all cases 96—99 per cent at rest, and unchanged during and after work. At rest in the supine position the oxygen saturation of the femoral venous blood was 53—81 per cent (mean 69) giving an AV oxygen difference of 23—85 ml/liter (mean 49). The venous oxygen saturation at rest was in good correlation to the pulse rate (Fig. 2). During the first load of exercise a venous oxygen saturation of 22—42 per cent (mean 30) was obtained at a heart rate of 68—102/min (mean 81). On continued exercise with successively increasing loads, a further slight but steady decrease in venous oxygen saturation occurred in every case, the lowest values always being recorded at the highest load, with minimal values of 13—34 per cent saturation (mean 21) at a heart rate of 159—187/min (mean 169) (Table I and Fig. 3).

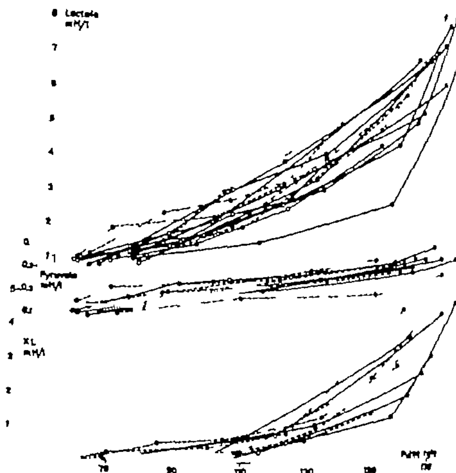


Fig. 5. Femoral venous lactate, pyruvate and "excess of lactate" (XL) in mM/liter at rest and and during exercise. Symbols as in Fig. 1

The venous oxygen saturation increased rapidly after work to values exceeding those at rest before work. Thus, the venous oxygen difference determined in the supine position 5 min after the end of exercise was 70—89 per cent (mean 79) the value being significantly higher than before work ($p < 0.001$). The initial level before exercise was regained about 15 min after the end of exercise.

There was no difference between the two age groups with respect to arterial or venous oxygen saturation at rest, during or after work.

pH

The pH of the femoral venous blood decreased during exercise from 7.41 at rest to 7.24 at maximal load (mean values). The corresponding values for arterial blood were 7.45 and 7.37. Still 5 minutes after work, the pH of both arterial and venous blood was lower than before work.

Table 1 Data obtained in connection with leg exercise in normal human subjects

		Rest	Heart rate during exercise				After exercise			
			90–110	110–130	130–150	150–175	5 min	10 min	15 min	30 min
Venous oxygen saturation, per cent	N	15	14	9	15	13	9	9	9	
	M	69	30	29	23	22	79	71	66	
	SE	2.1	1.4	1.9	0.6	1.0	1.8	2.8	2.8	
	SD	8.0	5.6	5.8	1.8	4.0	5.5	7.9	8.5	
Arterial pH	N	10	7	4		10	6			
	M	7.432	7.409	7.396		7.369	7.339			
	SE	0.004	0.006			0.011	0.011			
	SD	0.010	0.017			0.026	0.022			
Venous pH	N	10	7	4	5	10	7			
	M	7.418	7.343	7.327	7.297	7.239	7.311			
	SE	0.006	0.015			0.011	0.020			
	SD	0.021	0.038			0.026	0.052			
Venous pO_2	N	10	7	4		10	7			
	M	37	25	23		20	43			
	SE	1.1	0.7			0.6	1.7			
	SD	3.5	1.8			1.5	5.1			
Arterial lactate mM per liter	N	15	9	7	10	13	6		4	
	M	0.97	1.71	2.80	3.62	5.61	4.84		2.79	
	SE	0.03	0.10	0.22	0.18	0.41	0.42			
	SD	0.13	0.26	0.57	0.52	1.21	1.05			
Venous lactate mM per liter	N	15	11	9	10	15	10	8	5	5
	M	0.97	2.10	2.91	3.78	6.56	6.29	5.42	[illegible]	[illegible]
	SE	0.03	0.17	0.23	0.18	0.42	0.53	0.49	[illegible]	[illegible]
	SD	0.11	0.58	0.83	0.46	1.24	1.75	1.14	[illegible]	[illegible]
Arterial pyruvate mM per liter	N	10		10	6	10	10			
	M	0.11		0.18	0.21	0.25	0.39			
	SE	0.01		0.01	0.02	0.01	0.01			
	SD	0.02		0.02	0.04	0.05	0.05			
Venous pyruvate mM per liter	N	10		10	6	10	10	7		
	M	0.12		0.22	0.27	0.31	0.4	[illegible]	[illegible]	
	SE	0.01		0.01	0.01	0.02	0.03	[illegible]	[illegible]	
	SD	0.02		0.05	0.05	0.08	[illegible]	[illegible]		
Arterial XL mM per liter	N		7		7	10	16			
	M	0	0.16		0.38	2.84	[illegible]			
	SE		0.04		0.06	0.27	[illegible]			
	SD		0.09		0.15	0.86				
Venous XL mM per liter	N		9		8	10		5	[illegible]	
	M	0	0.26		1.03	3.1[illegible]	[illegible]		[illegible]	
	SE		0.05		0.07	[illegible]	[illegible]	[illegible]		
	SD		0.15		0.20	[illegible]	[illegible]	[illegible]		

Abbreviations N number of observations; M, mean SE standard error; SD standard deviation.

Blood oxygen tension

The oxygen tension of arterial and venous blood was calculated in 10 cases from the HbO_2 and pH, using the diagram of DILL, EDWARDS and CONSOLAZIO (1937) The resting value for arterial blood calculated on this basis was 85—115 mm Hg (mean 96) During light and moderate work, no significant change in arterial pO_2 was observed. At the end of work, however a slight decrease in the mean value to 90 mm Hg was observed, which agrees with earlier observations made with direct methods (HOLMGREN and LINDERHOLM 1958) The resting value for femoral venous pO_2 was 33—47 mm Hg (mean 37) A steady decrease in venous pO_2 to 20—25 mm Hg was found during the first two loads thereafter an essentially steady level was maintained during continued exercise (Table I) Five minutes after the end of work, the venous pO_2 had increased to 33—55 mm Hg (mean 47) The values for the mean capillary pO_2 during exercise, as calculated from BARCROFT's (1934) formula

$$\text{ven. } pO_2 + \frac{\text{art. } pO_2 - \text{ven. } pO_2}{3}$$ are plotted in Fig 3

Arterial and venous lactate concentration

No significant difference could be demonstrated at rest between the arterial and femoral venous lactic acid concentration, when calculated on the individual differences (0 75—1.23 mM/liter mean 0 97 and 0 79—1.25 mM/liter mean 0 97 respectively) As seen in Fig 4 and 5 and Table I a steady rise in both arterial and venous lactate was observed during the first loads, with a more pronounced increase at the highest load at which lactate concentrations of 4.35—7 73 mM/liter (mean 5 71) and 4 45—8.66 mM/liter (mean 6 56) respectively for arterial and femoral venous blood were recorded. This arterio-venous lactate difference is significant ($p < 0.01$)

) Five minutes after work the venous lactate concentration had further increased compared to the levels at the end of work in 3 cases, was constant in 3 cases and had slightly decreased in further 4 cases (Fig 6) The arterial lactate generally decreased more rapidly than the venous lactate. Five minutes after work, the mean arterial and venous lactate concentration was 4 81 and 6.29 mM/liter respectively and after 15 min 2.60 and 3 75 mM liter respectively These differences are highly significant ($p < 0.001$) There was no relation between the lactate concentration at maximal exercise and that 5 min after work a successive decrease in lactate was observed in cases with both high and low concentration at the end of work (Fig 6)

Arterial and venous pyruvate concentration

Pyruvic acid concentration at rest was 0 08—0.15 mM/liter (mean 0.11) in arterial blood, and 0 09—0 15 mM/liter (mean 0 12) in femoral venous blood and no significant AV difference was found. During exercise, a successive increase was observed in both arterial and venous blood, with a maximal value

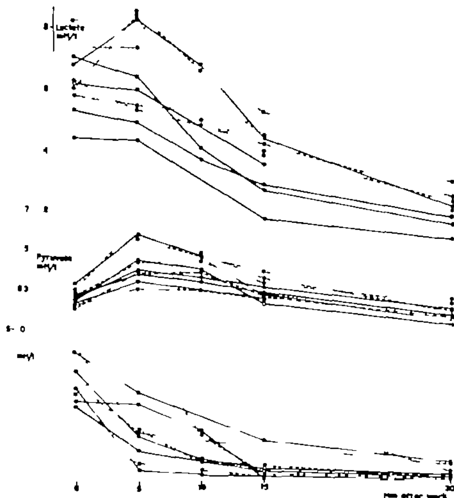


Fig. 6. Femoral venous lactate, pyruvate and XL   maximal work performed (time 0) and after exercise Symbols as in Fig. 1

of 0 33 mM/liter in venous blood t 900 kpm/minute. At the highest loads, the mean arterial and enous pyruvat concentrations were 0.25 and 0.31 mM/liter respectively the difference being significant ($p < 0.01$) When work stopped, the arterial and venous pyruvat increased further with a maximum reached 5 min after cessation of work at which moment   mean venous pyruvate of 0 45 mM liter was obtained. It then decreased successively and 30 min after work had fallen to bout the same level as at the end of work (Fig 6) During the first 30 min after the end of work the AV difference in pyruvate concentration was significant ($p < 0.01$)

Blood lactate-pyruvate ratio.

Excess of lactate (*XL*) was calculated according to the formula $X_L = (L - L_o) - (P - P_o)\left(\frac{L}{P}\right)$ where L and P are the resting values for lactate and pyruvate, and L_n and P the corresponding values at the different work loads (HOCKABEE 1958 a). During exercise, venous as well as systemic XL concentration closely followed those of the lactate, with the maximal increase at the highest loads (Fig. 4 and 5 and Table I). After work, the XL rapidly decreased in all but one case (Fig. 6).

In the present series no significant difference was present between the two age groups with respect to the lactate and pyruvate concentration at rest or during work. After work, however, the lactate concentration decreased more slowly in the older subjects than in the younger (Fig. 6), whereas interestingly enough no such trend was observed for the XL.

Discussion

With the technique used in this study it was possible to follow the oxygen utilization and the production of lactate and pyruvate during exercise. As pointed out in a previous paper (CARLSON and PERNOW 1959) it is probable — in view of the position of the venous catheter — that the greater part of the blood collected originated from muscle, at any rate during work. All data obtained during work have been plotted against the relative working intensity as expressed as the rise in pulse rate during exercise. This seems to be most adequate, since differences in physical working capacity can hereby be largely eliminated. This fact has most clearly been demonstrated by HOLMGREN and STRÖM (1959). They showed that patients with heart failure had a higher blood lactate concentration than healthy subjects when correlated to the absolute work (work load), whereas no difference was obtained when the lactate concentration was correlated to the relative work intensity (heart rate). In the present series, no significant difference was observed between subjects of various age with respect to the rise in heart rate during work (Fig. 1).

The femoral venous oxygen saturation at rest ranged from 33 to 81 per cent, and was in linear relation to the heart rate (Fig. 2) with the highest saturation at the highest heart rate. Since the oxygen uptake in the legs is probably constant at rest, the AV oxygen difference is inversely correlated to the pulse rate. Consequently, at a high heart rate, which normally indicates a large cardiac output, either a smaller relative amount of oxygen is taken up in the muscles, or a greater part of the blood flows through non-muscular tissues. The oxygen saturation of venous blood probably reflects the distribution of the blood flow through different tissues, rather than being a measure of the metabolic state (PAPPENHEIMER 1941).

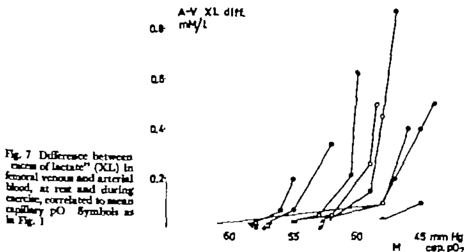


Fig. 7 Difference between "excess of lactate" (XL) in femoral venous and arterial blood, at rest and during exercise, correlated to mean capillary pO Symbols as in Fig. 1

Already during the first minutes of the initial load, the venous oxygen saturation had decreased to 22—42 per cent. This decrease is, however obviously not due entirely to the exercise, since a considerable decrease in oxygen saturation is produced merely by changing the position from supine to sitting (FLORKIN, EDWARDS and DILL 1940) This was confirmed in three cases in this series, in which the femoral venous saturation decreased from 65—73 to 24—31 per cent merely by changing the position from supine to sitting The further decrease during exercise was small. These changes at rest were obviously due to a variation in blood flow which has been shown to be markedly reduced when changing the body posture from supine to sitting (BEACONSFIELD and GINSBURG 1955) In two healthy subjects (not included in this series) two leg exercises were performed in the recumbent position. An immediate fall in femoral venous oxygen saturation was observed during work. The changes in the oxygen and lactate concentration in the blood were similar to those in sitting exercise, when correlated to the increase in heart rate. As shown in a previous paper (CARLSON and PERNOW 1959) a simultaneous decrease in venous oxygen saturation was also observed in resting tissues during one leg exercise. It was supposed that this decrease, which was of about th same magnitude as in th exercising tissue, reflected a decrease in blood flow through resting tissues during exercise.

It is interesting to note that the oxygen utilization was almost similar at different working intensities. Even during heavy work (heart rate up to 187/minute) the blood leaving the exercising leg was saturated to 21 per cen (mean value) It is not known whether more complete utilization takes pl during maximal work. The fact that the femoral enous blood contai siderable oxygen even on heavy work might be due either to the

that a certain amount of blood is still flowing through non-muscular tissues, or to incomplete oxygen uptake from muscular blood. As will be shown in the following paper in this series (CARLSON and PERNOW in press) practically complete oxygen utilization may occur during work under certain conditions, i. e., in patients with impaired peripheral circulation. Fig. 7 shows that, despite the fairly high oxygen tension of the blood on heavy work, the aerobic capacity is incomplete, as is evident from the rapid increase in XL formation.

The values obtained for arterial blood lactate were of the same order of magnitude as those presented earlier with the same analytic method (GOLDSMITH 1948) whereas even lower values have been reported by others (for references see HUCKABEE 1958 a) The values obtained for arterial pyruvate are also in agreement with earlier reports (GOLDSMITH 1948 and others) No significant difference was obtained at rest between the arterial and venous lactate concentration. This also applied to arterial and mixed venous blood (HOLMGREN 1959) whereas significantly higher values were observed in venous muscular blood of the forearm than in arterial samples (ANDRES, CADER and ZIERLER 1956) No significant difference was found between young and old subjects with regard to arterial or venous lactate concentration at rest or during exercise, which conforms with earlier observations (HOLMGREN and STRÖM 1959) In larger series, however a higher arterial lactate concentration in relation to heart rate during work has been observed in older healthy subjects than in younger ones (ÅSTRAND 1960) Obviously the similarity in rate of lactate accumulation, as well as of oxygen consumption and AV oxygen difference at different ages found in this study might indicate a similar increase in regional blood flow during exercise. A comparable blood flow through the calf at rest and immediately after work has, in fact, been found in young and older healthy subjects with the plethysmographic technique (ALLWOOD 1958)

After work, the lactate concentration decreased more slowly in the older subjects than in the younger (Fig 5) This fact cannot, however be taken as proof of prolonged tissue hypoxia since the same difference between the two age groups was observed with respect to pyruvate. The fall in XL factor after work was the same in the older subjects as in the younger ones (Fig 6)

As mentioned in the introduction, the lactate alone is not a definite index of the adequacy of cellular oxygenation since an increase in lactate is also observed on other occasions where the oxygen supply of the tissues is not decreased. The onset and degree of anaerobic work are best studied by simultaneous estimation of lactic and pyruvic acid concentrations in the blood (HUCKABEE 1958 a, b) During work, lactate alone is markedly raised in the arterial blood, whereas pyruvate is only slightly (ASMUSSEN 1950 HUCKABEE 1958 b) which — as shown in this study — also applies to venous blood from exercising tissue. Consequently the curves for total lactate and excess of lactate run practically parallel (Fig 4 and 5) During work, blood lactate determinations can therefore normally be regarded as a specific test of the effectivity of oxygen supply

to the tissue. This is not, however the case after work. As seen from Fig 6, the venous blood lactate either further rose, was unchanged or fell slowly 5 min after the end of exercise. This observation must not be taken as an indication of a prolonged state of tissue hypoxia. It was found that the pyruvate concentration consistently increased immediately after work, and that the lactate-pyruvate ratio therefore rapidly decreased, which is in conformity with earlier observations on arterial blood (ASMUSSEN 1950 HUCKABEE 1958 b)

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References

ALLWOOD, M. J Blood flow in the foot and calf in the elderly comparison with that in young adults. *Clin. Sci.* 1958. *17* 331—338.

ANDRES, R., G. CADER and K. L. ZIERLER, The quantitatively minor role of carbohydrate in oxidative metabolism by skeletal muscle in intact man in the basal state. Measurements of oxygen and glucose uptake and carbon dioxide and lactate production in the forearm. *J clin. Invest.* 1956. *35.* 671—682.

ASMUSSEN, E., Pyruvate and lactate content of the blood during and after muscular work. *Acta physiol. scand.* 1950. *20* 125—132.

ÅSTRAND, I., Aerobic work capacity in men and women with special reference to age. *Acta physiol. scand.* 1960. *49.* Suppl. 169. 1—92.

ÅSTRAND, P-O., Experimental studies of physical working capacity in relation to sex and age. *Copenhagen. Munksgaard.* 1952

BARCROFT J Features in the architecture of physiological function. *University Press*, Cambridge. 1934

BARKER, S. B. and W H. SUMMERSON, The colorimetric determination of lactic acid in biological materials. *J biol. Chem.* 1941 *138.* 535—554

BEACONSFIELD P and J GINSBURG, Effect of changes in limb posture on peripheral blood flow *Circ. Res.* 1955 *3.* 478—482.

BERNEUS, B., A. CARLSTEN, A. HOLMGREN and S. I. SELDINGER, Percutaneous catheterization of peripheral arteries as method for blood sampling *Scand. J clin. Lab Invest.* 1954. *6.* 217—221

BING, R. J., A. SIEGEL, I UNGAR and M. GILBERT Metabolism of the human heart. II. Studies on fat, ketone and amino acid metabolism *Amer J Med.* 1954. *16.* 504—515.

BISHOP J M., K. W DONALD, S. H. TAYLOR and P N. WORMALD, The blood flow in the human arm during supine leg exercise. *J Physiol.* 1957 *137* 294—308.

CARLSON, L. A. and B. PERNOW Oxygen utilization and lactic acid formation in the legs at rest and during exercise in normal subjects and in patients with arteriosclerosis obliterans. *Acta med. scand.* 1959 *164* 39—52.

CARLSON, L. A. and B. PERNOW Studies on the peripheral circulation and metabolism in man. II. Oxygen utilization and lactate-pyruvate formation in the leg at rest and during exercise in patients with arteriosclerosis obliterans. T be published.

DILL, D. B H. T EDWARDS and W V CONSOLAZIO, Blood as physicochemical system Man rest. *J biol. Chem.* 1937 *118.* 635—648.

DILL, D B., H. EDWARDS, A. FÖLLING, S. A. OBERG, A. M. PAPPENHEIMER TALBOTT Adaptations of the organism to changes in oxygen pr 1931 *71* 47—63.

DONALD, K. W., P. N. WORMALD, S. H. TAYLOR and J. M. BISHOP. Changes in the oxygen content of femoral venous blood and leg blood flow during leg exercise in relation to cardiac output response. *Clin. Sci.* 1957. *16*, 567—591.

DRABKIN, D. L., Measurement of O saturation of blood by direct spectrophotometric determination. Methods in Med. Res. 1950. 2. 159—161. *Year Book Publishers*, Chicago.

FLORKIN, M., H. T. EDWARDS and D. B. DILL, Oxygen utilization in the legs of normal men. I. The effect of posture. *Amer. J. Physiol.* 1930. *94*, 459—463.

FRIEDEMANN, T. E. and G. E. HAUGEN, Pyruvic acid. II. The determination of keto acids in blood and urine. *J. biol. Chem.* 1943. *147*, 415—442.

GOLDBERG, G. A. and A. FONTAINE, The blood lactate-pyruvate relationship in various physiologic and pathologic states. *Amer. J. med. Sci.* 1948. *215*, 182—188.

HOLMGREN, A., Arterio-venous lactic acid differences in man at rest and during muscular work. *Scand. J. clin. Lab. Invest.* 1955. *11*, 150—153.

HOLMGREN, A. and H. LINDERHOLM, Oxygen and carbon dioxide tensions of arterial blood during heavy and exhaustive exercise. *Acta physiol. scand.* 1958. *44*, 203—215.

HOLMGREN, A. and B. PERNOW, Spectrophotometric measurement of oxygen saturation of blood in determination of cardiac output. *Scand. J. clin. Lab. Invest.* 1959. *11*, 143—149.

HOLMGREN, A. and G. STRÖM, Blood lactate concentration in relation to absolute and relative work load in normal men, and in mitral stenosis, atrial septal defect and vasoregulatory asthenia. *Acta med. scand.* 1959. *163*, 185—193.

HUCKABEE, W. E., Control of concentration gradients of pyruvate and lactate across cell membranes in blood. *J. appl. Physiol.* 1956. *9*, 163—170.

HUCKABEE, W. E., Relationships of pyruvate and lactate during anaerobic metabolism. I. Effects of infusion of pyruvate or glucose and of hyperventilation. *J. clin. Invest.* 1958. *37*, 244—254.

HUCKABEE, W. E., Relationships of pyruvate and lactate during anaerobic metabolism. II. Exercise and formation of O_2-debt. *J. clin. Invest.* 1958. *37*, 255—263.

LILJESTRAND, G. and G. STRÖM, The concentration of blood lactic acid in man during muscular work in relation to the partial pressure of oxygen of the inspired air. *Acta physiol. scand.* 1947. *13*, 253—266.

PAPPENHEIMER, J. R., Vasoconstrictor nerves and oxygen consumption in isolated perfused hindlimb muscles of dogs. *J. Physiol.* (Lond.) 1941. *99*, 182—200.

SELIGSON, D. and B. SHAPIRO, Alpha-keto acids in blood and urine studied by paper chromatography. *Analyt. Chem.* 1952. *24*, 754—755.

STRÖM, G., The influence of anoxia on lactate utilization in man after prolonged muscular work. *Acta physiol. scand.* 1949. *17*, 440—451.

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Further Studies on the Effect of Catechol on the Isolated Guinea Pig Ileum

By

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Abstract

Sjöstrand, N. O. Further studies on the effect of catechol on the isolated guinea-pig ileum. Acta physiol. scand. 1961. 52. 343—349. — Catechol in doses of 0.1—2 mg added to the isolated guinea-pig ileum in a 15 ml bath usually induced a contraction of the strip. Atropine and paralyzing doses of cocaine and hexamethonium abolished this contraction. BOL and phenbenzamine had no effect on contractions due to catechol. Catechol in non-stimulating doses reduced the inhibition caused by adrenaline and noradrenaline. It is suggested that catechol like nicotine stimulates the gut through the mediation of acetylcholine and stimulation of the intramural ganglia.

Catechol has been found as a natural constituent of human urine (Fiker 1953, Euler and Lishajko 1959) and may therefore occur in some tissues and exert local physiological actions. In this respect the findings of Bacq (1936) that catechol exerts a sensitizing action on the effect of adrenaline, probably due to inhibition of the catechol o-methyltransferase (Bacq *et al.* 1959) are of great interest.

In a previous report the effect of catechol on the isolated guinea-pig ileum has been studied (Sjöstrand 1960). It was found to induce contraction of the ileum, when doses of 0.5—5 mg/15 ml were used, and to cause relaxation of the strip when bigger doses were used. Stimulation of plain muscles due to catechol has earlier been described by Barger and Dale (1910—11), who suggested a direct effect on the smooth muscle fibres. Since in the previous report the catechol induced contraction was abolished by atropine and by paralyzing doses



Fig. 1 Isolated guinea-pig ileum, bath volume 15 ml.
A = 0.01 μg acetylcholine, C = 0.3 mg catechol, H = 0.1 μg histamine, N = 5 μg nicotine, S = 0.5 μg serotonin. Between arrows 150 μg cocaine in the bath.

nicotine, the conclusion was drawn that catechol exerts its stimulating property on the isolated guinea-pig ileum through the mediation of acetylcholine and stimulation of the intestinal ganglia. The present study was undertaken in order to obtain further information on the above mentioned effect and to examine whether catechol could modify the actions of adrenaline and noradrenaline on the guinea pig ileum.

Materials and methods

Young guinea-pigs weighing 150—2[illegible]0 g were killed by a blow on the head. A strip of about 2 cm was taken from the caudal part of the ileum and fixed in a 15 ml organ bath containing Tyrode solution aerated with oxygen and kept at 38° C. The contrac-tions were recorded with a linear frontal writing lever.

The solutions used were acetylcholine chloride 0.01—10 μg per ml, adrenaline hydro-chloride *10—100* μg per ml, 2-brom-lysergic acid diethylamide (BOL) 50—500 μg per ml, catechol *1—10* mg per ml, cocaine hydrochloride 10 mg per ml, hexamethonium hydrobromide *1—10* mg per ml, histamine dihydrochloride 0.1—10 μg per ml, nicotine bitartrate 0.1—1 mg per ml, noradrenaline hydrochloride 10—100 μg per ml, phen-benzamine (*Lergitin*) 0.1—1 mg, serotonin creatinine sulphate 1—10 μg per ml and substance P 10 units per ml.

When the effect of catechol on the action of adrenaline and noradrenaline was examined the following procedure was used. A dose of acetylcholine, histamine or substance P giving steady responses was chosen, then the maximal dose of catechol that had no effect by itself or on the test dose was found. When this was done a dose of adrenaline or noradrenaline giving slightly more than fifty per cent decrease of the response to the test dose was given this dose was then given together with the catechol dose and the effect on the test dose was examined. The test doses were given [illegible]

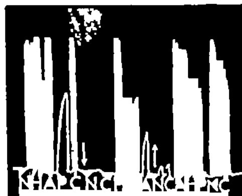


Fig. 2. Isolated guinea-pig ileum; bath volume 15 ml.
A = 0.03 μg acetylcholine, C = 0.25 mg catechol, H = 0.2 μg histamine, N = 10 μg nicotine, P = 0.5 units substance P. Between arrows 300 μg hexamethonium in the bath. About 5 minutes between each contraction.

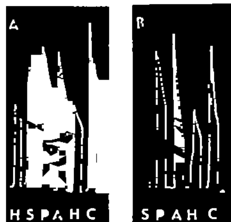


Fig. 3. Isolated guinea-pig ileum; bath volume 15 ml.
A = 0.01 μg acetylcholine, C = 0.5 mg catechol, H = 0.01 μg histamine, P = 0.5 units substance P. S = 0.5 μg serotonin.
A. before BOL.
B. 10 μg BOL in the bath.

interval of 5 min and the doses of adrenaline, noradrenaline and catechol were added to the bath one minute before the test doses.

In each series of experiment 5—10 experiments with intestines from 3—6 different animals were performed.

Results

Catechol in doses of 0.1—2 mg added to the 15 ml organ bath usually induced a contraction of the gut. The contraction often showed tachyphylaxis, which generally could be avoided if the intestine was allowed to rest for about 5 min between each catechol induced contraction. In some cases the earlier described after effect (SJÖSTRAND 1960) was seen.

Cocaine (10—50 μg/ml) inhibited the contraction due to catechol the contractions due to nicotine (5—20 μg) and serotonin (0.05—5



Fig 4. Isolated guinea-pig ileum bath volume 15 ml. A = 0.01 μg acetylcholine, C = 0.1 mg catechol, H = 0.01 μg histamine.
A. before phenbenzamine (Lergitin)
B. 10 μg phenbenzamine in the bath.

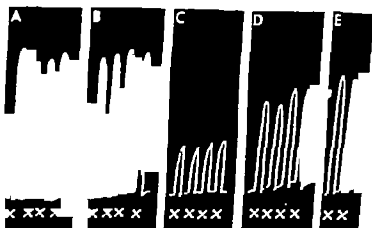


Fig 5. Isolated guinea-pig ileum bath volume 15 ml.
At × addition of 0.01 μg acetylcholine. 5 minutes between each contraction.
A. before adrenaline and catechol.
B. 20 μg catechol in the bath 1 minute before contraction.
C. 1 μg adrenaline 1 minute before contraction.
D. 1 μg adrenaline and 20 μg catechol 1 minute before contraction.
E. after adrenaline and catechol.

the effects of histamine, acetylcholine and substance P remained unaltered or were augmented (Fig 1)

Hexamethonium (5—200 μg ml) obliterated the effect of catechol and that of nicotine. The effects of substance P serotonin and histamine were unaffected or increased after addition of hexamethonium. The effect of acetylcholine was increased, unchanged or reduced (Fig 2

BOL in doses (5—50 μg) sufficient to greatly reduce the action of sero-

tonin (0 1—5 μg) had no effect on contractions caused by catechol acetylcholine, histamine and substance P (Fig 3)

Phenbenzamine (Lergitin) in doses of 1 to 10 μg which depressed the effect of histamine (0 01—1 μg) while leaving those of acetylcholine and substance P unimpaired, had no effect on contractions due to catechol (Fig 4)

Catechol in a non-stimulating dose (10—50 μg) added together with a depressing dose of adrenaline or noradrenalin (1—5 μg) reduced the inhibiting action of adrenaline and noradrenaline upon contractions caused by acetylcholine, histamine and substance P (Fig 5) When catechol was added to the bath together with adrenaline or noradrenaline there was sometimes seen an increase in the basal tone of the strip.

Discussion

It has been shown in the present study that the stimulating action of catechol on the isolated guinea-pig ileum can be abolished by cocaine and hexamethonium but not by BOL or phenbenzamine (Lergitin)

From the previous experiments (SJÖSTRAND 1960) it was suggested that catechol does not exert a direct tonic effect on the smooth muscle fibres as assumed by BARGER and DALE (1910—11) but stimulates the gut through liberation of acetylcholine mediated by the intramural ganglia, since the effect is abolished by atropine and paralysing doses of nicotine. Although atropine may be regarded as an almost specific parasympaticolyti drug and the used doses of nicotine probably merely acted on the intestinal ganglia, since they hardly did affect histamine induced contractions (which often are reduced by even moderate paralysing doses of nicotine) the presumed ganglionic site of action of catechol ought to be confirmed by other paralysing drugs.

Cocaine in the used concentrations is believed to act only on the nervous elements of an isolated ntestine, especially on the postganglionic nerves. The fact that cocain inhibits the effect of catechol as well as the effect of nicotine, which according to our present knowledge stimulates the ganglia of the gut, indicates a neural origin of the catechol induced contraction. The findings that cocaine inhib ts the effect of serotonin on the guinea-pig ileum agree with previous experience (ROCHA E SILVA, VALLE and PICARELLI 1953 GADDUM and PICARELLI 1957) Hexamethonium is regarded as a highly specific ganglionic blocking substance its obliteration of the contractions caused by catechol and nicotine forms a rather convincing support of the idea, that catechol, like nicotine stimulates the intramural ganglia The inhibiting effect of hexamethonium on cetylcholine produced contractions in some preparations may be explained by assuming that in these strips stimulation of ganglion cells partly contributed to the contraction due to acetylcholine, in agreement w the findings of *Feldberg* (1951)

It seems unlikely that catechol releases other smooth muscle stimulating substances than acetylcholine in the gut, such as histamine, serotonin or substance P. The effect of histamine is not inhibited by atropine or by moderate concentrations of cocaine or hexamethonium but is rather augmented; moreover the specific histamine antagonist phenbenzamine left the catechol induced contractions unimpaired. The serotonin induced contractions are not affected by hexamethonium, but by BOL, a potent serotonin antagonist (SOLLERO, PAGE and SALMOIRAGHI 1956) which in the present study had no effect on the contractions caused by catechol. The action of substance P is not antagonized by atropine, cocaine, hexamethonium, or nicotine. It might be of interest to compare the effect of catechol on the gut with its effect on the cardiovascular system, where it exerts a cardiostimulant action of sympathetic origin on the intact animal, whilst the effect on the isolated heart is depression (WALTON *et al.* 1959, GATGOUNIS and WALTON 1960).

In view of the findings of BACQ (1936) that catechol sensitizes peripheral organs to the effect of adrenaline, probably because it inhibits the catechol o-methyltransferase (BACQ *et al.* 1959), the antagonism seen in this investigation is rather puzzling. The effect of adrenaline and noradrenaline on the terminal part of the guinea-pig ileum is, however, not purely depressant: thus in preparations maintained in a state of tonic contraction by histamine, MUNRO (1953) found that small doses of adrenaline induced relaxation of the strip, while large doses caused a motor response. It might be possible to explain the present results by assuming that catechol through inhibiting the inactivation of adrenaline and noradrenaline causes a larger but less inhibiting concentration of the catechol amines in the intestinal tissue. In favour of such a view speaks the increase in basal tone of the strip sometimes seen. Another possible explanation is that catechol inhibits the action of adrenaline and noradrenaline through competition with peripheral receptors as found for sympathomimetic amines on the rabbit intestine (ÅSTRÖM 1949).

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References

BACQ, Z. M., Recherches sur la physiologie et la pharmacologie du système nerveux autonome. XX. Sensibilisation à l'adrénaline et à l'excitation des nerfs adrénergiques par les antioxygènes. *Arch. int. Physiol.* 1936. *42.* 340—366.

BACQ, Z. M., L. GOSSELIN, A. DRESSE and J. RENSON, Inhibition of o-methyltransferase by catechol and sensitization of epinephrine. *Science* 1959. *130.* 453—454.

BARGER, G. and H. H. DALE, Actions of amines. *J. Physiol.* (Lond.) 1910—11. *41.* 19—59.

EULER, U. S. v. and F. LISHAJKO, Excretion of catechol in human urine. *Nature* (Lond.) 1959. *183.* 1123.

FELDBERG, W. Effects of ganglion-blocking substances on the small intestine. *J. Physiol.* (Lond.) 1951. *113*, 483—493.

FÜRST, S., Paper chromatographic division of multivalent phenols. *Chem. Abstr.* 1955. *49*, 3734.

GADDUM, J. H. and Z. P. PICARELLI, Two kinds of tryptamine receptor. *Brit. J. Pharmacol.* 1957. *12*, 323—328.

GATGOUNIS, J. and R. P. WALTON, Resorcinol isomers and pentylenetetrazol: their centrally mediated sympathetic circulatory effects. *J. Pharmacol.* 1960. *128*, 363—371.

MUNRO, A. F. The effect of adrenaline and noradrenaline on the activity of isolated preparations of the gut from the foetal guinea-pig. *Brit. J. Pharmacol.* 1958. *8*, 38—41.

ROCHA E SILVA, M., J. R. VALLE and Z. P. PICARELLI, Pharmacological analysis of the mode of action of serotonin upon the guinea-pig ileum. *Brit. J. Pharmacol.* 1953. 378—388.

SJÖSTRAND, N., The effect of catechol on the isolated guinea-pig ileum. *Acta physiol. scand.* 1960. *49*, 57—61.

SOLLERO, L., I. H. PAGE and G. C. SALMOIRAGHI, Brom-lysergic acid diethylamide a highly potent serotonin antagonist. *J. Pharmacol.* 1956. *116*, 10—15.

WALTON, R. P., R. P. WALTON JR. and W. L. THOMPSON, Inotropic activity of catechol isomers and a series of related compounds. *J. Pharmacol.* 1959. *125*, 202—207.

ÅSTRÖM, A., Antisympathetic action of sympathicomimetic amines. *Acta physiol. scand.* 1949. *18*, 295—307.

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Influence of Various Factors on Urine Oxygen Tension in the Dog

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Abstract

AUKLAND, K. and J. KROG. *Influence of various factors on urine oxygen tension in the dog.* Acta physiol. scand. 1961 52. 350—365. — Urine pO_2 (U-pO_2) was recorded polarographically for 2—4 hours in 17 experiments on anaesthetized dogs. During air breathing values between 8 and 50 mm Hg were observed. *Histamine, papaverine and Regitin* invariably increased U-pO_2, with slight and inconstant changes in total renal blood flow (RBF), renal venous pO_2 (V-pO_2) and urine flow. Single doses of adrenaline and nor-adrenaline i. produced rapid, transient reduction of RBF, V-pO_2 and urine flow whilst U-pO_2 increased. Rapid infusion of hypertonic solutions of urea, mannitol or sodium chloride gave a rapid, transient increase of RBF whilst U-pO_2 was lowered for several minutes. Oxygen breathing produced a marked rise in V-pO_2, whilst U-pO_2 increased slightly or remained unchanged. It is suggested that U-pO_2 is determined by pO_2 in *the renal papilla through equilibration across the walls of the collecting* ducts. The main determinants are suggested to be 1 the rate of medullary blood flow 2 medullary oxygen consumption, possibly dependent on the load of sodium to the medulla. Plasma skimming and counter current diffusion of O_2 in the medulla is discussed. The findings are consistent with an independent regulation of cortical and medullary blood flow.

It has been demonstrated by several investigators that the oxygen tension (pO_2) of urine is far lower than the oxygen tension of renal venous blood (SARRE 1937, HOSO and RAHN 1957, RENNIE, REEVES and PAPPENHEIMER 1958). The explanation of this finding is not clear. Lowering of urine pO_2 by reducing substances in urine before measurement can be carried out was excluded as

the cause by REHORIK et al. (1958) The same investigators showed also that at low urine flow a high degree of equilibration with respect to O may be reached between urine flowing through the ureter and venous blood in the ureteral wall. They found, however that the error introduced by gaseous exchange in the urinary tract was small when urine flow was higher than 5 ml/min, and demonstrated clearly that the pO of urine leaving the kidney is low (6—60 mm Hg, mean 28 mm)

In anesthetized dogs REHORIK et al. (1958) frequently observed spontaneous variations of urine pO_2, sometimes as rhythmical variations. These spontaneous variations could not be correlated to changes in blood pressure or renal venous pO_2, and it was therefore suggested that urine oxygen tension is dependent upon a labile mechanism governing intrarenal distribution of blood flow" It was therefore of interest to find out how urine pO is influenced by vasoactive substances. Furthermore, possible correlations between urine pO_2, arterial blood pressure, total renal blood flow and renal venous pO was investigated.

Methods

I all 17 experiments were performed in 15 healthy mongrel dogs of either sex, weighing from 15—25 kg Anesthesia was induced by Nembuthal-sodium, 25 mg/kg body weight. Smaller doses of Nembuthal-sodium were added throughout the experiment for maintenance of light anesthesia. The first experiments revealed, however that doses of 2—3 mg/kg body weight or more, often produced temporary increase of urine pO_2. In subsequent experiments maintenance doses were therefore reduced to 1—2 mg/kg

All animals were intubated to insure free air ways. Most experiments were performed with spontaneous respiration of air I some experiments the effect of oxygen respiration was studied by administration of pure oxygen through an automatic respirator

The ureter on one side was exposed by flank incision and cannulated with a polyvinyl catheter The tip of the catheter was placed at the pelviureteral junction, and the other end connected to the oxygen electrode chamber A permanent draining catheter was introduced into the bladder to prevent accumulation of urine from the other kidney

Urine pO

The presence of reducing substances in urine and the relatively rapid equilibration with systemic tissue pO_2 in the urinary tract necessitates rapid registration device with minimum of dead space for continuous pO_2 recording The polarographic method was therefore selected as the most suitable for the problems to be investigated.

The oxygen tension was measured by means of polarographic electrode similar to that described by KROG and JOHANSEN (1959) The electrod was mounted in plastic chamber thermostatically controlled by circulating water through an outer jacket. The total olume of the electrode chamber and the catheter was less than 1 ml. To obtain an electrode assembly with rapid response to pO changes, teflon membrane with thickness of 0.00025 was used as covering This, however resulted in an assembly slightly sensitiv to flow Calibration of the electrode was therefore done before and after each experiment with saline equilibrated with gas mixtures with known O concentration, with flow rate corresponding to the average urine flow during the experim

Within the flow range of 2—15 ml/min employed the error introduced by flow sensitivity was less than 5 %. The "rest current" of the instrument during passage of nitrogen-equilibrated saline was only 2—4 % of the current registrated when aerated saline (21 O_2) was flowing past the electrode. The stability of the system was satisfactory. Continuous reading of urine pO was obtained for 2—4 hours in each of 17 experiments, with less than 10 % drifting in the course of any experiment.

Renal venous pO

In 3 experiments the oxygen tension of venous blood was recorded continuously by a similar electrode mounted on a heart catheter (KROG and JOHANSEN 1959) introduced into the renal vein through a jugular vein. As the position of the electrode within the lumen could not be controlled, artifacts produced by blunting against the venous wall could not entirely be avoided. For the same reason the calibration was probably less exact than for the urine electrode.

Renal blood flow

In 5 experiments renal blood flow was measured continuously by an electromagnetic square wave flowmeter[1]. The probe was applied directly on the isolated renal artery. In another 6 experiments effective renal plasma flow was estimated by clearance of para-amino hippuric acid (PAH) in periods of 5—10 min. Arterial blood for PAH determination was drawn in the middle of each clearance period. Urine and plasma PAH was determined by the method of SMITH *et al.* (1945).

Arterial pressure

A brachial artery was cannulated with a polyethylene catheter which was connected to a Statham strain gauge transducer. Continuous pressure recording was obtained by means of a Sanborn recording system.

Infusions

All experiments were performed during high urine flow in order to ensure oxygen equilibration between the urine and the renal pelvis.

In two experiments diuresis was induced by rapid i. v. infusion of 0.45 or 0.9 saline. In all other experiments osmotic diuresis was produced by i. v. infusion of mannitol (5 to 15 or urea 5 or 10 in 0.45 saline, at a constant rate of 8 ml/min. In 6 experiments PAH was added to the infusion in amounts to provide a constant plasma concentration in the region between 0.5 and 2 mg/100 ml. In addition 0.45 saline was given by drip at a rate adjusted according to urine flow to compensate for excessive salt and water loss. Urine flow was measured frequently at the outlet of the electrode chamber by means of graded vials.

Administration of drugs

In two experiments weak solutions of drugs were infused directly into the renal artery through a thin polyethylene catheter introduced via the left carotid artery. In all other experiments drugs were infused into a brachial vein cannulated with a polyethylene catheter. Continuous infusions were provided by an electric infusion pump calibrated to deliver 0.25, 0.8 or 1.5 ml/min.

[1] Manufactured by Kiper Dennard Associates, Winston-Salem, N. C., U. S. A.



Fig. 1 Effect of Nembuthal i.v. on urine pO_2. Dog ♂ 23 kg Mannitol diuresis, 12 ml/min.

Results

Control values and spontaneous variations in urine pO

During respiration of air urine pO varied between 8 and 50 mm Hg. This large scattering was not only due to individual variations, as it was repeatedly observed that a high urine pO at the start of the recording might change to low levels in the course of the experiment. Alteration in the opposite direction was also seen several times. Spontaneous increase of urine pO by 5—15 mm Hg over a period of 2—5 min appeared in several experiments, at times more or less regularly with an interval of 5—10 min. These variations could not be correlated to changes in arterial pressure, total renal blood flow renal venous pO_2 or respiration.

Changes in urine flow within the limits studied (2—15 ml/min) seemed not to influence urine pO in any consistent manner Neither was any consistent difference found between mannitol diuresis, hypertonic salt diuresis or water diuresis. However our experience with the latter two types of diuresis is limited to two experiments.

High spontaneous urine pO was often associated with low arterial pressure (mean pressure below 100 mm Hg) while low urine pO was most often recorded during high blood pressure. However exceptions to this rule were observed rather frequently

Effect of anesthesia

When anesthesia became insufficient as indicated by shivering movements of the limbs and irregular increase of arterial pressure, urine pO tended to decrease. Maintenance doses of Nembuthal-sodium, 3—5 mg/kg body weight then generally produced a transitory increase of urine pO_2. The response was generally less than that shown in Fig 1 However in some experiments a sustained increased level of urine pO was observed after a single dose of Nembuthal.

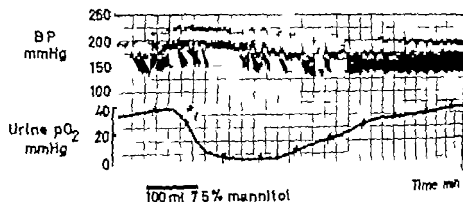


Fig. 2. Effect of rapid i. v. infusion of 7.5 % mannitol in 0.9 % sodium chloride on arterial blood pressure and urine pO_2. Dog ♀ 14 kg. Mannitol diuresis produced by continuous i. v. infusion of 7.5 % mannitol at rate of 8 ml/min. Urine flow: Control 8 ml/min. 2 min after rapid infusion 9 ml/min. 8 min after infusion 7.5 ml/min.

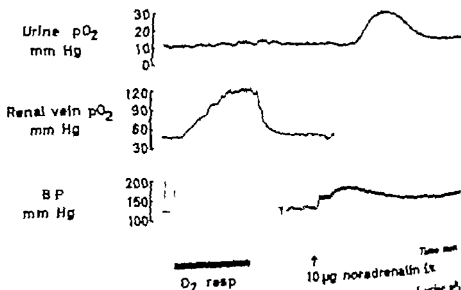


Fig. 3. Effect of oxygen respiration on mean arterial pressure, renal venous and urine pO_2 compared to the effect of i. v. infusion of nor-adrenaline. Dog ♂ 26 kg. Mannitol diuresis 10 ml/min.

Hypertonic infusions

Rapid intravenous infusion of 20—100 ml 7.5—30 mannitol, 10 urea or 5 saline produced a temporary change of urine pO. Most often a slight increase of urine pO of less than 1 min duration was followed by a marked decrease lasting for 5—10 min, while renal blood flow increased considerably for a period of less than one minute (Fig. 2).

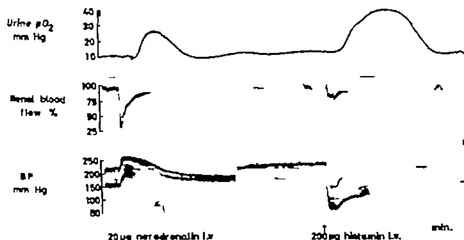


Fig. 4. Effect of i. injection of nor-adrenaline and histamine on arterial pressure, renal blood flow and urine pO_2. Dog ♀, 20 kg. Manitol-urea diuresis, 9 ml/min. Sharp deflection on flow curve at right indicates clamping of renal artery for calibration. (First half of BP curve damped by clot in arterial catheter.)

Effect of breathing pure oxygen

When urine pO was lower than 20 mm Hg changing between air and oxygen respiration produced no significant change in urine pO_2, whilst renal venous pO increased to high levels during oxygen breathing (Fig 3). At higher levels of urine pO_2, oxygen breathing produced a moderat increase of urine pO starting after approximately 1 min and reaching maximal value in 4—6 min. In several experiments, however spontaneous variations in urine pO were larger than those produced by changing between air and oxygen respiration.

Effect of adrenaline and nor-adrenaline

A single i. v. injection of 10—50 µg drenaline or nor adrenaline produced invariably (10 injections in 7 different dogs) a transient increase of urine pO_2, starting 1—2 min after the injection. Maximum was observed after 2—6 min, and pre-injection level was reached again after 3—10 min (Fig 3 4 7). In several instances a slight decrease in urine pO of less than 1 min duration preceded the increase. Renal blood flow fell by 25—75 % following the injection and then returned to normal or slightly supernormal values after 1—2 min. The oxygen tension of renal venous blood showed either no change at all or a small decrease of less than 1 min duration. Urine flow decreased markedly immediately following the injection and then returned to pre-injection values or somewhat higher within 1—2 min. The increase in urine pO might thus subsist for several minutes after normalization of total renal blood flow renal vein pO and urine flow



Fig. 5 Effect of i. v injection of Regitin on arterial pressure and urine pO_2. Effect of nor-adrenaline partially blocked by Regitin. Dog ♂ 12.5 kg Mannitol diuresis, 6 ml/min.

Injection of adrenaline or nor-adrenaline directly into the renal artery in so small doses that almost no systemic effects could be observed, gave principally the same response of urine pO_2 as described above for i. v administration. It was thus shown that the effect of these substances was direct on the kidney and not mediated via reflexes or produced by alterations in arterial pressure.

Continuous i. v infusion of adrenaline or nor-adrenaline gave varying results. When urine pO_2 was low doses of 5—30 µg/min produced an irregular increase of urine pO_2. When infused at higher level, however both substances tended to cause a decrease of urine pO_2. At intermediate levels the response was quite unpredictable and could not be related to infusion rate.

Continuous infusion of dilute solutions of adrenaline or nor-adrenaline into the renal artery tended to depress urine pO_2. However also by this mode of administration the response was variable. The intraarterial infusion also reduced urine flow renal blood flow and renal venous pO_2.

No principal difference between the renal effect of adrenaline and nor-adrenaline were observed.

Effect of Regitin[1]

In 3 experiments Regitin was injected i. v in doses producing only slight reduction of arterial blood pressure (10—25 mm Hg) A marked, sustained increase of urine pO_2 was observed in all experiments following the administration of 3—4 mg Regitin while renal blood flow did not change appreciably (Fig 5) Renal venous pO_2 was not measured in these experiments. Regitin reduced the effect of adrenaline and nor-adrenaline on urine pO_2, as shown in fig. 5.

Effect of papaverine

A single i. v injection of 4—20 mg papaverine produced regularly (6 injections in 3 different dogs) a transient increase of urine pO_2, lasting for 3 to 7 min. This was the case even when the doses employed were too small to

[1] Regitin "Ciba" (N-(p-Tolyl-N-oxyphenylaminomethyl)-imidazoline methansulfonate).

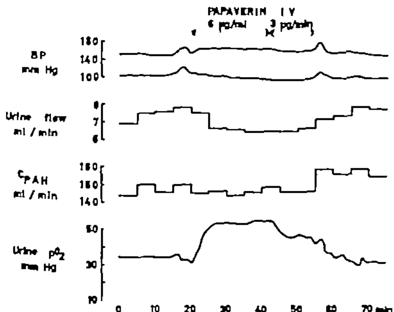


Fig. 6. Effect of continuous i. v. infusion of papaverine on arterial pressure, urine flow, PAH clearance and urine pO_2. Dog ♀ 22 kg. Mannitol diuresis.

produce ppreciable alteration in mean arterial pressure, total renal blood flow or renal venous pO_2.

Continuous i. v. infusion of papaverine at a rate of 2—12 mg/min during 3 periods of 25 to 35 min duration in two dogs, raised urine pO to high levels (40—55 mm Hg). The response was clearly related to the infusion rate (Fig. 6).

Effect of histamine

Rapid, single injections of histamine i. v. produced regularly (8 injections in 6 dogs) a marked increase of urine pO_2, starting within $/_2$—1 min after the injection. Maximal values were reached after 2—3 min and pre-injection level was reached after 5—10 min. When doses of 200 µg histamine were used, mean arterial pressure and renal blood flow fell to varying extent for 1—2 min, while renal venous pO decreased by 10—20 mm Hg for an even shorter period of time (Fig. 4 7). With smaller doses, however, these parameters remained practically unaltered, while urine pO increased significantly.

By continuous i. v. infusion of histamine it was possible to maintain urine pO at high levels for seemingly indefinite periods of time. This was consistently found in all 10 infusion periods of 13—65 min duration in 6 different dogs. It was shown by varying the infusion rate between 8 and 60 µg/min that t increase of urine pO was clearly related to the dosage of histamine. A



Fig. 7 Effect of i. v. injection of histamine on urine pO_2, renal venous pO_2 and arterial pressure compared to the effect of nor-adrenaline. Dog ♂ 26 kg. Mannitol diuresis 13 ml/min.

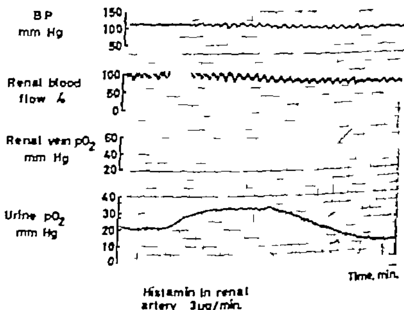


Fig. 8. Effect of histamine infusion into renal artery on arterial pressure, renal blood flow, renal venous and urine pO_2. Dog ♂ 18 kg. Mannitol diuresis. Urine flow gradually declining from 5.0 to 3.9 ml/min.

fall of arterial blood pressure and sometimes also a decrease of renal blood flow was observed during the infusion. By infusion of histamine directly into the renal artery at a rate of 1.5—10 μg/min it was shown, however, that the increase of urine pO_2 could be obtained independently of changes in arterial pressure, renal blood flow and renal venous pO_2 (Fig. 8).

Among the drugs tried in this study histamine was found to be the most reliable agent to initiate and maintain an increase in urine pO_2.

Discussion

The oxygen consumption of the kidney is low compared to the large amount of oxygen made available by the high rate of blood flow to this organ. The oxygen tension of renal venous blood is therefore high, generally in the range of 60—70 mm Hg The abundance of capillaries in the renal cortex should prohibit any great oxygen tension gradient from capillaries to renal tissue, and it would therefore be expected that the oxygen tension in cortical interstitial water should be nearly as high as in renal venous blood. Furthermore, it has been *calculated from Krogh-Hill's diffusion equation that the pO gradient* across the tubular wall should be less than 2 mm Hg (RENNIE *et al.* 1958) If the urine pO is determined in the convoluted tubules situated in the renal cortex it would therefore be assumed to have values approximately as high as renal venous blood. In agreement with earlier results, the present experiments have shown that this is not the case. It was found that urine pO is always lower than renal venous pO_2, and furthermore that urine pO can be varied quite independently of renal venous pO_2. These seemingly contradictory statements therefore demand an explanation.

It might be suggested that a large proportion of the renal blood flow is shunted through arterio-venous anastomoses, thereby providing only small amounts of oxygen to the renal tubules, simultaneously producing a high oxygen content of renal venous blood. While this hypothesis might explain some of the findings regarding urine oxygen tension, it is incompatible with the high extraction ratio of substances like diodrast or PAH, as stressed by SMITH (1951) It should also be pointed out that a.-v shunts have never been anatomically demonstrated in the kidney in spite of careful search (TRUETA *et al.* 1947 VON KÜGELGEN *et al.* 1959)

Based on extensive studies in anesthetized dogs, RENNIE *et al.* (1958) suggested that the low urine pO is due to plasma skimming providing low hematocrit blood and thereby blood with low oxygen capacity to the peritubular circulation (PAPPENHEIMER and KINTER 1956) Assuming that the degree of skimming could be estimated by the PAH extraction ratio, RENNIE *et al.* (1958) found that urine pO varied inversely with the degree of plasma skimming The same authors also presented experimental evidence to show that the low urine pO can not be explained by a failure of equilibration across the tubular wall. It should be pointed out, however that a failure of equilibration would probably not cause a low urine pO_2, because it must be assumed that the pO of the glomerular filtrate is nearly as high as in arterial blood (about 100 mm Hg during air breathing) Oxygen must therefore diffuse out from the tubular lumen to an area with even lower pO than that of pelvic urine.

As an alternative explanation RENNIE *et al* (1958) mentioned that the urine pO *might be determined in the collecting ducts. By rather laborat calculations* based on several assumptions it was found, however that the urine

in this case must be equilibrated with tissue supplied with low hematocrit blood. They suggested therefore that a skimming process might provide low hematocrit blood to the medullary circulation. The anatomical circumstances would probably favour an equilibration in the collecting ducts as well as in the convoluted tubules, and several authors have *a priori* assumed that urine pO_2 is determined in this place (SARRE 1938, ULLRICH 1959, LEVY 1959, KRAMER *et al.* 1960). If this hypothesis is correct, it follows that the papillary interstitial pO_2 must be very low, even lower than that of pelvic urine, because the direction of oxygen diffusion must be out from the lumen (*s.s.*). The interstitial oxygen tension in the various zones of the kidney is not known. In a previous study it was shown, however, that the mean oxygen tension is lower in the medulla than in the cortex, compatible with a low pO_2 in the tissues surrounding the collecting ducts (AUKLAND and KROG 1960).

The following factors might be suggested as responsible for a low papillary oxygen tension:

1. Low papillary blood flow.
2. Counter current diffusion of oxygen across the loops of the vasa recta.
3. Alkaline pH within the papilla, with resulting high hemoglobin oxygen binding capacity at low pO_2.
4. Plasma skimming providing low hematocrit blood to the medullary circulation.
5. High medullary oxygen consumption.

Some aspects of these factors will be discussed in the following.

1. Small amounts of oxygen may be brought to the papilla by urine both *via* the loops of Henle and *via* the collecting ducts. The flow rate of urine in the loops of Henle and the flow rate of blood in the vasa recta have been shown to be of the same order of magnitude (LILIENFIELD, BAUER and MAGANZINI 1959, KRAMER *et al.* 1960). However, due to the low oxygen capacity of urine compared to that of blood it must be assumed that the main oxygen supply is *via* the vasa recta, thus depending on the flow rate in these vessels. The extraordinary length of the medullary capillaries (vasa recta) would *per se* indicate a low oxygen supply to the medulla, and various estimations have also shown that the blood flow itself is low as compared to cortical blood flow. A slow accumulation of thorotrast in the rabbit medulla and of labelled albumin in the papilla of dogs has been demonstrated (TRUETA *et al.* 1947, LILIENFIELD *et al.* 1959). SCHIEVE *et al.* (1959) found by thermometric methods that the blood flow in the inner portions of cortex was 25 times greater than in the middle of the inner medulla. Similar results were obtained by measurement of dye appearance time by KRAMER *et al.* (1960), who stated that the blood flow of the papilla per g tissue was about one twentieth of that in the renal cortex. The concept of a relatively low blood supply to the renal medulla as compared to that of the cortex seems thus well established.

2. It has also been suggested that the oxygen supply to the papilla is further limited by the counter current flow in the vasa recta (LEVY 1959) It must be assumed that oxygen will diffuse from the descending to the ascending portion of the vessel loops according to pO_2 gradients. The quantitative significance of this shortcircuiting of oxygen will therefore be greatest when arterial pO_2 is high as during oxygen breathing, and even greater during oxygen breathing at 2.5 atm. pressure, as used in the experiments of RENNIE *et al.* (1958) As mentioned by ULLRICH (1959) it is possible that this mechanism invalidates the calculation of the dynamic hematocrit of the region where urine pO_2 is determined (RENNIE *et al.* 1958)

The extent to which the counter current exchange diffusion will reduce the supply of oxygen to the medulla will depend upon the rate of oxygen diffusion across the vessel loops and on the flow rate in the vessels. In short time experiments no changes in the diffusion coefficient would be expected. The oxygen supply to the medulla should therefore depend upon the blood flow in such a manner that an increase of blood flow would result in more than proportional increase of oxygen supply

3 The pH of papillary interstitial fluid is not known. The low effective blood flow to the medulla with respect to highly diffusible substances would favour the accumulation of acid metabolites. On the other hand, secretion of hydrogen ions into the collecting ducts within the papilla (ULLRICH, EIGLER and PEHLING 1958) should tend to increase pH of interstitial fluid surrounding the collecting ducts. The pH might thus be expected to be determined by the relative magnitude of these two opposing factors. In dogs breathing oxygen, KRAMER *et al.* (1960) found no increase of papillary hemoglobin oxygen saturation after injection of cyanide into the renal artery They concluded therefore that the blood in the medullary circulation is nearly completely saturated with oxygen during oxygen breathing It may be questioned, however if a single injection of cyanide will reach the inner medulla in sufficient amounts to stop respiration effectively as it must be assumed that the penetration of cyanide into the inner medulla will be greatly impeded by the counter current exchange mechanism. As far as we can see, this finding is only compatible with the very low urine pO_2 if blood within the papilla is strongly alkaline compared to systemic blood. On the other hand, the slight or lacking effect on urine pO_2 of oxygen breathing would indicate that the hemoglobin in the papilla is not nearly saturated with oxygen.

Although we can not exclude that an alkaline reaction within the papilla may contribute to provide a low urine pO_2, it seems quite unlikely that the acute variations in urine pO_2 produced by drugs should result from variations in papillary pH.

4 Plasma skimming providing low hematocrit blood in the medullary circulation can not be excluded by the present experiments. It was repeatedl shown, however that great variations in urine pO_2 could be produced wit

significant alterations in arterial pressure or renal blood flow factors which should determine the degree of skimming. The cell separation theory thus seems insufficient to explain our findings.

5. The oxygen consumption of the inner medullary zone (including the papilla) has been found to be low compared to cortical oxygen consumption, both *in vitro* (For references see ULLRICH 1959) and *in vivo* (KRAMER *et al.* 1960). Evidence has also been presented to show that the papillary oxygen consumption is directly dependent on the hemoglobin oxygen saturation within the papilla (KRAMER *et al.* 1960). The effect of variations in papillary blood flow on urine pO_2 should therefore be buffered by a varying rate of oxygen consumption in the papilla.

The oxygen consumption of the outer medullary zone has been shown to be high compared to that of the inner zone (For references see ULLRICH 1959). As the capillary loops of the inner medulla also traverse the outer medullary zone, the oxygen consumption of the outer zone might determine pO_2 in the inner zone, and thereby also urine pO_2. It has recently been suggested that the main part of renal oxygen consumption is concerned with, and parallels tubular sodium reabsorption (HESS THAYSEN, LASSEN and MUNCK 1960, KRAMER and DEETJEN 1960, KIIL, AUKLAND and REFSUM 1961). The demonstration of a low sodium concentration in the first part of the distal convoluted tubules (GOTTSCHALK and MYLLE 1959) shows that sodium is avidly reabsorbed in the loops of Henle, and then probably by the wide portion of the ascending limb of the loops. ULLRICH and PEHLING (1958) found that the oxygen consumption of slices from the outer medulla increases linearly with sodium concentration in the medulla in the range of 0—450 meq/l, and suggested that this was due to increased sodium transport by the cells of the wide ascending limb, which is localized in the outer medullary zone. It is thus possible that an increased load of sodium to the wide ascending limb, brought about by increased glomerular filtration rate or by osmotic diuresis, might increase sodium transport and oxygen consumption of the outer medullary zone, and thereby reduce papillary and urine pO_2.

From the general discussion above it appears that acute changes in urine pO_2 is most likely due either to changes in papillary blood flow and/or to changes in outer medullary oxygen consumption. If papillary blood flow is the main determining factor, the present findings show that *papillary blood flow* may be varied widely independent of the cortical blood flow. (For practical purposes the total renal blood flow may be taken as a measurement of cortical flow, as the cortical circulation probably receives about 98 % of the total blood supply to the kidney (KRAMER *et al.* 1960).)

An independent regulation of cortical and medullary blood flow is by no means a new hypothesis. TRUETA *et al.* (1947) found in rabbits subjected to various traumatic procedures that the cortical circulation might decrease markedly while the flow apparently increased in the medulla. This finding of

diversion of cortical blood flow to the medulla through the juxtamedullary glomeruli has been confirmed by several authors in rabbits, but has not been demonstrated in dogs (For references see SMITH 1951) Recently however THURAU *et al.* (1960) found that the medullary blood flow of the perfused dog kidney varied proportional to perfusion pressure, while cortical flow showed autoregulation, *i. e.* constant flow at increasing perfusion pressure. In the present experiments no consistent correlation between blood pressure and urine pO was found, probably due to a direct effect of the drugs on the medullary circulation.

GRUPP (1957) studied the distribution of renal blood flow by temperature measurements in the dog kidney High doses of adrenaline i. v (40 μg/kg) stopped the renal circulation for 1—2 min. In the following 5—6 min, however total renal blood flow nearly reassumed control values, while cortical renal blood flow was still greatly reduced. He concluded that during these conditions a high proportion (at least 50 %) of the normal renal blood flow might pass the kidney in spite of ischemic renal cortex. In the present study a marked increase of urine pO following injection of adrenaline or noradrenaline might directly suggest an increased medullary blood flow It is difficult to offer any pharmacological explanation to this finding, however While it is well known that adrenaline may produce vasodilatation, no such effect has been recorded for nor-adrenaline (GOODMAN and GILMAN 1955) As the nor-adrenaline preparation used contained less than 4 % adrenaline, it is unlikely that the increase of urine pO following injection of nor-adrenaline was due to admixture of adrenaline. This was also indicated by nearly identical response to equal doses of adrenaline and nor-adrenaline. As we would hesitate to propose a vasodilatating effect of nor-adrenaline in the medullary circulation, it seems more reasonable to suggest that the rise of urine pO is due to a temporary reduction of glomerular filtration rate with resulting decrease of oxygen consumption in the outer medullary zone. It might also be suggested that the variable response to adrenaline and nor-adrenaline results from the interaction between two antagonistic effects 1 Increased vascular resistance in the medullary circulation, reducing blood flow and 2. Reduced oxygen consumption due to reduction of glomerular filtration rate.

The increase of urine pO following injection of adrenalin and nor adrenaline might also be explained on a hemodynamic basis if these substances had a larger vasoconstrictor effect on the cortical than on the juxtamedullary circulation. Reduction of blood flow to the cortex would increase the side pressure in the interlobular arteries and thus increase perfusion pressure in the afferent arterioles of juxtamedullary glomeruli, originating at right angles from the first portion of th intralobular arteries (TRUETA *et al.* 1947) Medullary blood flow would thus vary inversely to cortical blood flow A temporary increase in systemic hematocrit due to emptying of splenic erythrocyte reservoirs might

possibly also contribute to the increase in urine pO_2 observed after adrenaline and nor adrenaline injections.

The consistent increase of urine pO_2 resulting from vasodilatating drugs such as histamine, papaverine and Regitin, without concomitant increase of total renal blood flow would suggest a high vasoconstrictor tone in the medullary circulation in anesthetized dogs, in contrast to the cortical circulation. The same explanation might also apply to the increase of urine pO_2 produced by Nembuthal. Although variations in sodium load to the medulla might contribute to change urine pO_2 also in these experiments, it has been shown that papaverine and histamine may produce a marked increase of urine pO_2 with slight or no decrease of the filtered load of sodium (AUKLAND in preparation). The changes brough about by these agents are therefore most likely due to changes in the rate of medullary blood flow.

The decrease of urine pO_2 resulting from rapid intravenous infusion of hypertonic urea or mannitol can probably not be explained as resulting from increased vascular resistance with lowering of medullary blood flow. This is unlikely because hypertonic solutions generally increase blood flow and especially since it has been shown that papillary plasma flow increases during mannitol diuresis (KRAMER *et al.* 1960). On the other hand, mannitol and urea probably increases the amount of sodium which is brougth to the thick ascending limb of the loops of Henle, and might thereby increase O_2 consumption, either by increased sodium reabsorption or by a higher oxygen demand by the sodium reabsorbing mechanism in the presence of mannitol or urea (KIIL *et al.* 1961).

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References

AUKLAND, K. and J. KROG, Renal oxygen tension. Nature (Lond.) 1960. *188*. 671.

GOODMAN, L. S. and A. GILMAN, The pharmacological basis of therapeutics. *Second edition. New York, The Macmillan Company* 1955. pp 476.

GOTTSCHALK, C. W. and M. MYLLE, Micropuncture study of the mammalian urinary concentrating mechanism. Evidence for the counter current hypothesis. *Amer. J. Physiol.* 1959. *196*. 927—936.

GRUPP, G., Physiologische Betrachtungen über die Durchblutung der Niere. In: von Kügelgen, A., B. Kuhlo, W. Kuhlo and Kl. J. Otto. Die Gefässarchitektur der Niere. Zwangl. Abhandl. aus d. Gebiet d. norm. u. patholog. Anatomie. *Stuttgart, Georg Thieme Verlag* 1959. *Heft 5.* 1—7.

HESS THAYSEN, J., N. A. LASSEN and O. MUNCK, Sodium transport and oxygen consumption in the mammalian kidney. *Excerpta med.* (Amst.) Int. Congr. 1960. 29. 44.

HONG, S. K. and H. RAHN, Total and partial gas tensions of human urine. *Fed. Proc.* 1957. *16*. 61.

KIIL, F., K. AUKLAND and H. E. REFSUM, Renal sodium transport and oxygen consumption. *Amer. J. Physiol.* 1961 (In press.)

KRAMER, K. and P. DEETJEN, Sodium reabsorption and oxygen consumption of the kidney. *Excerpta med.* (Amst.) Int. Congr. 1960. 29. 30.

KRAMER, K., K. THURAU and P. DEETJEN, Hämodynamik des Nierenmarkes. I. Mitteilung. Capilläre Passagezeit, Blutvolumen, Durchblutung, Gewebshämatokrit und O_2-Verbrauch des Nierenmarkes in situ. *Pflügers Arch. ges. Physiol.* 1960, *270*, 251—269.

KROG, J. and K. JOHANSEN, Construction and characteristics of teflon-covered polarographic electrode for intravascular oxygen determination. *Rev. Sci. Instrum.* 1959, *30*, 108—109.

VON KÜGELGEN, A., B. KUHLO, W. KUHL and KL. J. OTTO, Die Gefässarchitektur der Niere. *Zwangl. Abhandl. aus d. Gebiet d. norm. u. patholog. Anatom.* Stuttgart, Georg Thieme Verlag 1959. *5*, 9—111.

LEVY, M. N. and G. SAUCEDA, Diffusion of oxygen from arterial to venous segment of renal capillaries. *Amer. J. Physiol.* 1959, *196*, 1336—1339.

LILIENFIELD, L. S., M. H. BAUER and H. C. MAGANZINI, Plasma flow in renal medulla. *Circulation* 1958, *20*, 729.

LOVOLEY, J. B., N. A. LASSEN and L. S. LILIENFIELD, Tracer studies on renal medullary circulation. *Fed. Proc.* 1958, *17*, 99.

PAPPENHEIMER, J. R. and W. B. KINTER, Hematocrit ratio of blood within mammalian kidney and its significance for renal hemodynamics. *Amer. J. Physiol.* 1956, *185*, 377—390.

RENNIE, D. W., R. B. REEVES and J. R. PAPPENHEIMER, Oxygen pressure in urine and its relation to intrarenal blood flow. *Amer. J. Physiol.* 1958, *195*, 120—132.

SARRE, H., Untersuchungen über Sauerstoff- und Kohlensäure-spannung im Harn und ihre Beziehung zum Nierengewebe und zur Nierenfunktion. *Pflügers. Arch. ges. Physiol.* 1938, *239*, 377—399.

SCHRIVE, J. F., J. W. CARHART, R. N. WATMAN and R. W. STOW, The distribution of blood flow in the kidney of the dog. *J. Lab. clin. Med.* 1959, *54*, 943—944.

SCHMIDT-NIELSEN, B., K. J. ULLRICH, R. O'DELL, G. PEHLING, C. W. GOTTSCHALK, W. E. LASSITER and M. MYLLE, Micropuncture study of the composition of fluid from cortical nephrons in the rat kidney. *Excerpta med.* (Amst.) Int. Congr. 1960, *29*, 72.

SMITH, H. W., The kidney. Structure and function in health and disease. New York, Oxford University Press 1951 pp. 626.

SMITH, H. W., N. FINKELSTEIN, L. ALIMINOSA, B. CRAWFORD and M. GRABER, The renal clearance of substituted hippuric acid derivatives and other aromatic acids in dog and man. *J. clin. Invest.* 1945, *24*, 388—404.

THURAU, K., P. DEETJEN and K. KRAMER, Hämodynamik des Nierenmarkes. II. Mitteilung. Wechselbeziehung zwischen vasculären und tubulären Gegenstromsystem bei arteriellen Drucksteigerung, Wasserdiurese und osmotische Diurese. *Pflügers Arch. ges. Physiol.* 1960, *270*, 270—285.

TRUETA, J., A. E. BARCLAY, P. M. DANIEL, K. J. FRANKLIN and M. M. L. PRICHARD, Studies of the renal circulation. Springfield, *Charles C. Thomas, Publ.* 1947.

ULLRICH, K. J., Das Nierenmark. Struktur, Stoffwechsel und Funktion. *Ergebn. Physiol.* 1959, *50*, 433—489.

ULLRICH, K. J., F. W. EIGLER and G. PEHLING, Sekretion von Wasserstoffionen in den Sammelrohren der Säugetierniere. *Pflügers Arch. ges. Physiol.* 1958, *267*, 491—496.

ULLRICH, K. J. and G. PEHLING, Aktiver Natriumtransport und Sauerstoffverbrauch in der äusseren Markzone der Niere. *Pflügers Arch. ges. Physiol.* 1958, *267*, 207—217.

Acta physiol. scand. 1961 52. 366—371

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Oxygen Uptake and Pulse Rate while Running with Undetermined and Determined Stride Lengths at Different Speeds

By

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Abstract

KNUTTGEN, H. G *Oxygen uptake and pulse rate while running with undetermined and determined stride lengths at different speeds* Acta physiol. scand. 52 366—371 — Two well-trained subjects ran to steady-state on a motor-driven treadmill at speeds between 9 and 16.5 km/h with undetermined stride lengths and at speeds between 9 and 11.66 km/h with a determined length of stride. Determinations of oxygen uptake and pulse rate were made during steady-state at each speed. The results indicate that kinetic energy is the predominant factor in running (a nearly rectilinear relationship was obtained between oxygen uptake and velocity to the second power) The increase in pulse rate was nearly rectilinearly related to that of oxygen uptake.

Certain references are found in the literature that report a rectilinear relationship between oxygen uptake and speed of treadmill running in steady-state (CHRISTENSEN and HÖGBERG 1950 HÖGBERG 1952 a) BOJE (1944) reported, however a curvilinear relationship between the two factors, with the oxygen uptake increasing at a proportionately faster rate with increasing speed. As a great part of the energy expenditure in running is presumably kinetic ($E = \frac{1}{2} mv^2$) in nature, one might expect that velocity to the second power would affect the oxygen requirement and oxygen uptake during running so as to cause a non-rectilinear relationship.

The results of various experiments on level walking support this contention. Most recently RALSTON (1958) and COTES and MEADE (1960) have reported that, not only is the relationship between energy expenditure and walking velocity curvilinear in nature but that, if energy expenditure is plotted against the square of velocity a rectilinear relationship is obtained. BOBBERT (1960) has arrived at the same conclusions but refers to the latter *relationship as* "nearly rectilinear"

In the present work it was postulated that there were two dominating factors involved in the energy cost of running on the level (1) the factor of kinetic energy and (2) the subject's adjustment of stride lenght in order to adapt himself to each speed. In an effort to determine the effects of these factors on steady-state oxygen uptake and pulse rate, experiments were conducted in which a subject ran at various speeds (a) determining his stride length for himself and (b) a parallel series where he used a pre-determined and unchanging length of stride at the various speeds.

Methods

Two well-trained runners (V K., age — 22 yrs., ht. — 170 cm, wt. — 74.5 kg and P K., age — 23 yrs., ht. — 179 cm, wt. — 73 kg) were employed as the test subjects in the experiments. The testing was conducted on motor-driven treadmill, the subject running continuously for approximately 10 min at each speed. He was considered t be in steady-state after 6 min, at which time recording of pulse frequency and collections of expired air were made. Pulse frequency was recorded by means of an electrocardiograph, one electrode being attached to the subjet' chest over the lower portion of the sternum, another to the subject' forehead, and third lead serving as ground. Expired air collected by the Douglas bag method, was analyzed according to SCHOLANDER (1947) Two tests were made on each day with a short period of rest between the two bouts of exercise. The order of running speeds was mixed with respect to high and low speeds so as to minimize any effects of conditioning or fatigue. The experiments were run between 11:00 a. m. and 1:00 p. m. the subject having had nothing to eat since a light breakfast prior to 8:00 a. m.

Three sets of experiments were conducted, one set with subject P K. and two with subject V K. (with a six month *interval between the latter two)* Since the *results of* the experiments were nearly identical, only the data from the second set of experiments with subject V K. are presented.

The experiments involving the two running techniques were run concurrently The experiments began by testing the subject at a speed of 9 km/h, the lower limit of both series, and his stride length was determined by counting the number of steps per minute and converting accordingly The subject was tested t eleven different speeds between 9 and 16.5 km/h determining stride length for himself ("undetermined" stride lengths) and t nine speeds between 9 and 11.66 km/h in the "determined" series. The lowest speed was selected as approximating the lower limit of true running while the upper limit was set at 16.5 km/h, the highest value at which true steady-state could be obtained. The subject ran at all speeds in the "determined series with the same stride length as was determined at 9 km/h (77 cm) by having him run in time to metronome appropriately adjusted to give the proper number of beats, and thus, strides, per minute. J

Table I Oxygen uptake, pulse rate, and stride length at various speeds while running with undetermined and pre-determined stride lengths

Speed (km/h)	VO_2 (l/m)	Pulse (/m)	Stride length (cm)
(Undetermined Series)			
9.00	2.46	140	77
9.75	2.61	141	83
10.50	2.75	143	90
11.25	[illegible].91	148	95
12.00	3.11	160	102
12.75	3.36	168	111
13.50	3.62	176	115
14.25	3.87	179	125
15.00	3.93	183	129
15.75	4.17	188	140
16.50	4.24	190	145
(Pre-determined Series)			
9.00	2.46	140	77
9.33	2.52	144	77
9.66	2.63	147	77
10.00	2.83	154	77
10.33	3.02	164	77
10.66	3.28	168	77
11.00	3.50	175	77
11.33	3.78	180	77
11.66	3.99	188	77

was not possible for the subject to run at speeds higher than 11.66 km/h with strides of 77 cm because of his inability to move the body parts quickly enough to maintain the extremely rapid tempo.

Results

The oxygen uptakes and pulse rates obtained are presented in Table I. When plotted in direct relationship to running speeds, the results for the "undetermined" series were found to present slowly rising curves up to a speed of 14 km/h after which the curves began rounding off as the subject neared maximal values. However plotting the values against velocity to second power (v^2) as shown in Fig. 1 produced quite different results. In the "undetermined" series a relatively rectilinear relationship was found between oxygen uptake and running velocity (to the second power) up to approximately 14 km/h. As speed was increased above this point, the relationship became definitely curvilinear in nature with the curves rounding off at the higher values. The relationships between pulse rate and velocity to the second power were "s" shaped, with pulse rate increas-

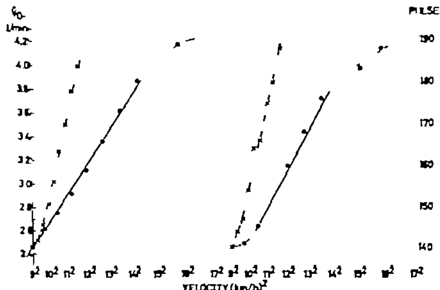


Fig. 1 Oxygen uptake and pulse rate while running with determined ( ) and undetermined ( ) stride lengths.

ing proportionately faster up to a speed of approximately 14 km/h and after this point, proportionately slower as indicated by the declining slope. In the case of the "determined" series, a linear relationship was found between velocity to the second power and oxygen uptake from 9.66 to 11 66 km/h. The values for oxygen uptake at 9 0 and 9.53 km/h were significantly different from theoretical values obtained from an extension of this linear relationship. The total relationship, therefore, took the form of a "J" shaped curve. A similar relationship was found between velocity to the second power and pulse rate.

Discussion

The fact that plotting oxygen uptake against velocity to the second power resulted in a nearly perfect rectilinear relationship for speeds between 9 and 14 km/h would indicate that running is almost entirely a matter of kinetic energy This would agree with the results of similar investigations in walking In the present study a curvilinear relationship was found at the highest level of running speeds, beginning above 14 km/h. The increase in oxygen uptake (and pulse rate) became smaller per unit increase in running velocity It is postulated that, because n running the subject is approaching maximal values, the body response to the demands of exercise becomes more and more retarded. It should be mentioned that, while the relationship between oxygen uptake while running in the "undetermined" series and velocity to the second power was "nearly recti-

in nature, it was not perfectly rectilinear It is suggested that, in addition to the factor of kinetic energy one or more other factors, such as lift work or additional body movements, enter in and that their relationships should be determined by future investigations.

The energy requirements of running with determined strides slightly shorter than the optimal, as in the lower speeds employed, showed little or no variation from the values obtained while running with undetermined stride lengths. Above 9 66 km/h, however there occurred a rapid and linear increase in values with the total curve being 'J' shaped. Högberg (1952 a) has shown that, when a subject runs at a certain speed and shortens and lengthens his stride length from the optium length, minor deviations cause only a slight change in energy cost while increasingly higher energy demands come about with increasingly larger deviations in stride length. In the present study the deviations from optimal lengths became increasingly greater at increasingly higher running velocities, thus causing the 'J' shaped relationship between oxygen uptake and running velocity

When pulse rate was plotted against oxygen uptake for the various speeds in both the "determined" and the "undetermined" series, nearly rectilinear relationships were obtained. With pulse rate as the ordinate and oxygen uptake as the abscissa, the points plotted for the "determined" series lay slightly higher with respect to pulse rates at various rates of oxygen uptake than did those of the "undetermined" series. One explanation might be a possible state of emotional excitement on the part of the subject at having to run in time to a metronome and at unnatural stride lengths.

With regard to the various curves obtained, the values that seem to be a bit higher than one might expect are those for pulse rate for the lowest running speeds in the "undetermined" series. The reason for this is not understood, but it is suggested that one cause could be the hydrostatic effect, which the increased milking action of the muscles helps to overcome at the higher running speeds.

The increase in stride length of the subject was found to be directly related to the increase in running speed in the "undetermined" series. The rectilinear relationship obtained from this test series coincided with the rectilinear relationship obtained from a single experiment undertaken as a check. The subject increased his velocity from 9 km/h to 16,5 km/h by increasing his stride length and not by increasing his stride frequency (there was actually a slight decrease in frequency)) These two results, the relationship of stride length to running speed and the subject's method of increasing his velocity are both in agreement with those of Högberg (1952 a) The results of Ogasawara (1934) also indicated an increase in stride length as the means by which a runner increases running speed, but a relatively large scattering of values was reported. The consistency of the results of the present study and of Högberg are quite likely due to the greater controls of experimental design and the degree of training of the subjects.

References

Bobbert, A. C., Energy expenditure in level and grade walking. *J. appl. Physiol.* 1960. *15* 1015—1201.

Bøje, O. Energy production, pulmonary ventilation, and length of steps in well-trained runners working on a treadmill. *Acta physiol. scand.* 1944. *7* 362—375.

Christensen, E. H. and P. Högberg, Steady-state O_2-deficit and O_2-debt at severe work. *Arbeitsphysiologie.* 1950 *14* 251—254.

Cotes, J. E. and F. Meade, The energy expenditure and mechanical energy demand in walking. *Ergonomics.* 1960 *3.* 96—119.

Högberg, P. How do stride length and stride frequency influence the energy-output during running? *Arbeitsphysiologie.* 1952 a. *14* 437—441.

Högberg, P. Length of stride, stride frequency, "flight" period and maximum distance between the feet during running with different speeds. *Arbeitsphysiologie.* 1952 b. *14* 431—436.

Ogasawara, M., Energy expenditure in walking and running. *J. Physiol.* (Lond.) 1934 *81* 255—264.

Ralston, J. J., Energy-speed relation and optimal speed during level walking. *Inter. Z. angew. Physiol.* 1958. *17* 277—283.

Scholander, P. F. Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. *J. biol. Chem.* 1947 *167* 235—250.

Acta physiol. scand. 1961 52. 372—378

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Cholinesterases in Human Spinal Fluid and Brain[1]

By

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Abstract

SVENMARK, O Cholinesterases in human spinal fluid and brain. Acta physiol. scand. 1961 52. 372—378. — By electrophoresis and chromatography on DEAE-cellulose it was shown that human spinal fluid and brain tissue extracts contained three cholinesterases Two butyrylcholinesterases and one acetylcholinesterase. One of the butyrylcholinesterases had the same electrophoretic mobility as serum cholinesterase. Th other butyrylcholinesterase moved together with the γ-globulin and the acetylcholinesterase together with the τ-globulin fraction of spinal fluid. These findings indicate that the acetylcholinesterase in spinal fluid derives from brain whereas it can not be decided whether the butyrylcholinesterases originate from plasma or from brain. The low mobility fraction of butyrylcholinesterase may arise from the action of sialidase.

Human spinal fluid catalyzes the hydrolytic splitting of benzoylcholine and acetyl-β-methylcholine indicating the presence of butyrylcholinesterase (pseudo-cholinesterase, unspecific cholinesterase) and of acetylcholinesterase (specific cholinesterase) The rates of splitting are about 1/200 of the rates at which the choline-esters are split by human plasma and erythrocytes (GLASOV and MUTRUX 1946, REISS and HEMPHILL 1948)

In human brain both types of cholinesterases occur (ORD and THOMPSON 1952) the butyrylcholinesterase is probably located in glia and Schwann cells (CAVANAGH THOMPSON and WEBSTER 1954) In rat brain acetylcholin-

[1] Preliminary report SVENMARK (1958)

esterase is associated with the cell structure and only small amounts occur in solution. Butyrylcholinesterase is found mainly in the cytoplasma (HOLMSTEDT and TOSCHI 1959). The acetylcholinesterase in spinal fluid might thus derive from brain and the butyrylcholinesterase from either brain or plasma or from both. A comparison of the electrophoretic mobility and chromatographic behavior of cholinesterases might indicate whether there are differences between the enzymes occurring in the spinal fluid, brain and plasma, i. e. whether spinal fluid cholinesterases can originate from plasma or brain.

Materials and Methods

Samples of *human spinal fluid* were obtained by lumbar, ventricular and cisternal puncture from neurosurgical, neurological and psychiatric patients. Small samples (5—15 ml) were concentrated at 0° C by vacuum dialysis (MIES 1953) and larger samples or pools by dialysis against 30 per cent polyethylene glycol (Carbowax 20 M, approximate m. w. 20,000, KOHN 1959).

Human brain tissue was obtained 24 hours after death (traffic accidents (3 brains), coronary occlusion (1 brain)). Grey matter, white matter and mixed tissues from one hemisphere were cut into small pieces (0.5 ml), rinsed in icecold 0.9 per cent sodium chloride and blotted on filter paper. The tissues were homogenized in a Waring blendor with icecold 0.9 per cent sodium chloride containing 40 mM/l NaH_2PO_4 adjusted to pH 7.4 with 0.1 N NaOH (1 liter per kg wet weight of the tissue). The homogenates were kept for one hour at 0° C and centrifuged at 8,000 × *g*. The extraction was repeated twice on the precipitate, and the supernatants were combined. The turbidity of the extract could be reduced by freezing to — 20° C and subsequent thawing and centrifugation. This procedure was repeated three times and the extract finally centrifuged half an hour at 30,000 × *g*. The supernatant was concentrated against 30 per cent carbowax 20 M, dialysed for 24 hours against the buffers used in electrophoresis and chromatography and adjusted to a protein concentration of 30 mg/ml.

Human serum was obtained from donors without signs or symptoms of disease.

Paper electrophoresis was performed with an LKB apparatus (LKB Fabriksaktiebolag, Stockholm) with SCHLEICHER & SCHÜLL no. 2043 bmgl filter paper and sodium barbital buffer (pH 8.6, μ = 0.075). 5 V/cm were applied for 18 hours at room temperature. Protein was stained with amidoblack 10 B and the electrophorogram was recorded on a paper strip scanner with logarithmic output.

Column electrophoresis was carried out on a column (15 × 400 mm) of ethanolyzed cellulose (PORATH 1954, FLODIN and KUPKE 1956) in sodium barbital buffer (pH 8.6, μ = 0.075); the eluate was collected in 1.5 ml fractions and the protein concentration determined (LOWRY *et al.* 1951).

For *chromatography* on diethylaminoethyl-cellulose (DEAE-cellulose, EASTMAN KODAK, SOBER *et al.* 1956) the protein was dialyzed against 5 mM NaH_2PO_4 adjusted to pH 7.0 with 0.1 N NaOH. Two ml were applied to the column (10 × 120 mm) and eluted at 5° C with a gradient of increasing salt concentration and decreasing pH (from 5 mM sodium phosphate buffer pH 7.0 to 0.5 M NaCl and 50 mM NaH_2PO_4). The eluate was collected in 5 ml fractions and the concentration of protein determined from the optical density at 280 mμ (Beckman spectrophotometer, model DU). The conductivity and the pH of the effluent were determined (Conductivity Meter CDM 2 and pH Meter PHM 22, Radiometer, Copenhagen).

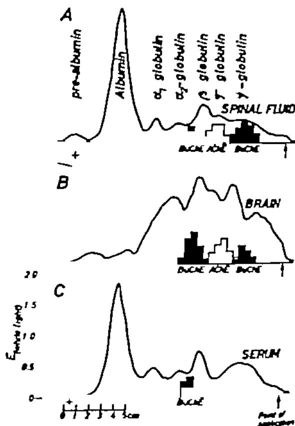


Fig. 1 Paper electrophoresis at pH 8.6 of (A) human spinal fluid, (B) human brain extract (mixed tissue) and (C) human serum. The height of the black columns denotes the activity of butyrylcholinesterase and the height of the open columns that of acetylcholinesterase.

To localize *cholinesterase activity* in paper electrophoresis the wet paper strips were cut into 5 mm transverse sections these were further cut into small pieces and 0.5 ml of 50 mM NaH PO (adjusted to pH 7.4) and 50 µl of 48 mM butyrylcholine iodide or acetyl-β-methylcholine chloride were added. The mixture was incubated at 37° C for one hour when butyrylcholine served as substrate and for 8 hours when acetyl-β-methylcholine was used. After the incubation period 450 µl of water were added and the concentration of the choline ester was determined colorimetrically (Hestrin 1949). Corrections for the spontaneous hydrolysis of the substrates were obtained from samples prepared with water in lieu of enzyme. Eluates from column electrophoresis or from column chromatography were assayed by the same method 450 µl of the sodium phosphate buffer and 50 µl of the substrate solution were added to 50 µl of the eluate and the assay performed as described above.

To compare the cholinesterase fractions isolated by column electrophoresis their enzymatic activities with different substrates and inhibitors were determined. Benzoylcholine chloride and butyrylthiocholine iodide were used in final concentrations of 4 mM. As inhibitors prostigmin bromide and 10-(α-diethylaminopropionyl) phenothiazine chloride (ASTRA 1397 Astra, Södertälje, Sweden) were used. The inhibitor was dissolved in the 50 mM sodium phosphate buffer and incubated with the sample for 30 min before the substrate was added.

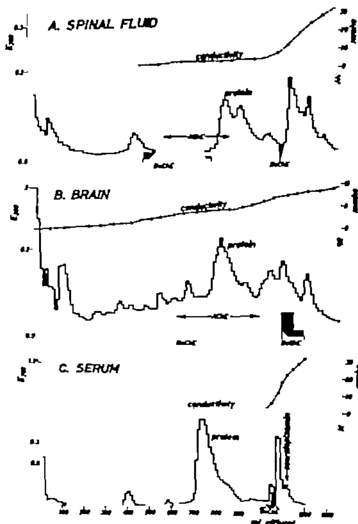


Fig. 2. Chromatography on DEAE-cellulose of (A) human spinal fluid, (B) human brain extract (mixed tissue) and (C) human serum. The height of the black columns indicates the activity of butyrylcholinesterase. The locations of acetylcholinesterase and greenish protein (coeruloplasmin) are indicated in the figure.

Results

Spinal fluid. Paper electrophoresis at pH 8.6 of concentrated spinal fluid (also 50 mg protein per ml) showed three fractions of cholinesterase. Two butyrylcholinesterases and one acetylcholinesterase. One butyrylcholinesterase was located between α_2 and β-globulin (the α-β-type) and the other together with γ-globulin (the γ-type). The ratio of the activities of the two

Table I Comparison of the enzymatic activity of the γ-type and the α-β-type butyrylcholinesterases of brain extracts (pH 7 4 37° C, substrate concentration 4 mM)

Substrate and inhibitor	Cholinesterase activity[1] (µmoles/(hour ml))		Relative cholinesterase activity[2]	
	γ-type	α-β-type	γ-type	α-β-type
Butyrylcholine	3.01	4.39	1 00	1.00
Butyrylthiocholine	2.36	3.19	0.78	0.73
Benzoylcholine	0.38	0.76	0.13	0.17
Acetyl-β-methylcholine	0	0.02	0	0
Butyrylcholine + prostigmin (4 10^{-6} M)	1.92	2.96	0.64	0 67
Butyrylcholine + ASTRA 1397 (6·10^{-6} M)	2.66	3.90	0.89	0 89

[1] duplicat determinations.
[2] ratio of activity with butyrylcholine as substrate to activity with the substrate in question.

butyrylcholinesterases varied from 1 2 to 2 1 in different samples. The acetylcholinesterase activity was associated with protein located between the butyrylcholinesterases or approximately together with the τ-globulin (Fig 1 A) This distribution of cholinesterases was seen in 22 different samples of spinal fluid and in 7 pools of spinal fluid.

In chromatography on DEAE-cellulose (Fig 2 A) one butyrylcholinesterase appeared together with γ- and β-globulins electrophoresis of this fraction showed that it was identical with the ν-type. The other butyrylcholinesterase appeared after albumin and was identical with the α β-type. Both butyrylcholinesterases hydrolyzed butyrylcholine but not acetyl β-methylcholine and were inhibited by prostigmin and ASTRA 1397 The acetylcholinesterase was eluted between the butyrylcholinesterases.

Brain tissue Paper electrophoresis of the brain extracts showed the same distribution of the cholinesterases as spinal fluid One butyrylcholinesterase appeared at a site corresponding to that of the α-β-type of spinal fluid and another together with γ-globulin Acetylcholinesterase appeared between these fractions of butyrylcholinesterase (Fig 1 B) This distribution of cholinesterases was seen in electrophoresis of extracts from four brains (mixed tissues) and from grey and from white matter of two brains.

The distribution of the cholinesterases in the DEAE-cellulose chromatogram of brain extracts was the same as in spinal fluid (Fig 2 B)

The two butyrylcholinesterases were isolated by column electrophoresis and the enzyme activity was measured with butyrylcholine, butyrylthiocholine benzoylcholine and acetyl β-methylcholine as substrates and with prostigmin and ASTRA 1397 as inhibitors. The activities (calculated in units of the activity measured with butyrylcholine as substrate) were identical in the α-β-type and the γ-type butyrylcholinesterase (Table I)

Serum. The electrophoretic mobility of the serum cholinesterase (GREGOIRE and DERRIEN 1952) corresponded to that of the α-β-type butyrylcholinesterase from spinal fluid and brain (Fig 1 C) In chromatography on DEAE-cellulose (Fig 2 C) human serum cholinesterase appeared between albumin and a greenish protein (coeruloplasmin)

Discussion

The butyrylcholinesterase and acetylcholinesterase activities of spinal fluid and brain were associated with different protein fractions. The electrophoretic mobility (pH 8.6) and the chromatographic behavior of the acetylcholinesterase in spinal fluid and in brain were the same. This suggests that part of the spinal fluid protein derives from the brain.

Two butyrylcholinesterases w th different electrophoretic mobilities occurred in spinal fluid and brain extracts. The α-β-type had the same electrophoretic and chromatographic properties as plasma cholinesterase and, therefore, it remains undecided whether the occurrence of this enzyme in spinal fluid is due to plasma or brain proteins. The low mobility butyrylcholinesterase, the γ-type, which occurs in both spinal fluid and brain extracts was absent in plasma. This is another indication that spinal fluid contains proteins deriving from brain. It is, however possible that the γ-type butyrylcholinesterase is produced by the action of sialidase which is present in spinal fluid (SVENNERHOLM 1961 a, b) The activity of sialidase is so low that its effect on the cholinesterases during the experiments can be neglected. *In vivo* sialidase might reduce the electrophoretic mobility of an α-β-type butyrylcholinesterase secreted from plasma or brain into the spinal fluid. This would give rise to the occurrence of a series of butyrylcholinesterase fractions with mobilities varying between that of the α-β-type and that of the γ-type. That only two butyrylcholinesterase fractions were bserved is not necessarily inconsistent with this explanation. On account of the low sialidase activity the amounts of butyrylcholinesterase with inter mediate mobilities might be too small to be detected by the procedures used in this study whereas the non-modified α-β-type and the accumulated sialic acid-free butyrylcholinesterase (the γ-type) might be present in a sufficiently large amount to be determined. Similarly the occurrence of two cholinesterases with dentical enzymatic properties but with different electrophoretic mobilities found in milk and colostrum of swine (AUGUSTINSSON and OLSSON 1959) might be due to the presence of sialidase.

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References

Augustinsson, K. B. and B. Olsson, Esterases in the milk and blood plasma of swine. I Substrate specificity and electrophoretic studies. *Biochem. J* 1959 *71* 477—484.

Cavanagh, J B., R. H. S. Thompson and G R. Webster, The localization of pseudo-cholinesterase activity in nervous tissue. *Quart. J exp. Physiol.* 1954 *39* 185—197

Flodin, P and D W Kupke, Zone electrophoresis on cellulose columns. *Biochim. Biophys. Acta* 1956 *21* 368—376

Glasson B. and S. Mutrux, Contribution à l'étude des cholinestérases dans le liquide céphalo-rachidien. *Helv physiol. pharmacol. Acta.* 1946. *4* 12—c 14

Grégoire, J and Y Derrien, Répartition de l activité cholinestérasique dans les fractions protéiques des sérum d'homme et de cheval séparées par électrophorèse sur papier *C. R. Soc Biol.* (Paris) 1952. *146* 1491—1493

Hestrin, S The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. *J biol. Chem.* 1949 *180* 249—261

Holmstedt B. and G. Toschi, Enzymic properties of cholinesterases in subcellular fractions from rat brain. *Acta physiol. scand.* 1959. *47* 280—283

Kohn J A simple method for the concentration of fluids containing protein. *Nature* (Lond.) 1959 *183* 1055

Lowry O H., N J Rosebrough, A. L. Farr and R. J Randall, Protein measurement with the Folin phenol reagent. *J biol. Chem.* 1951 *193* 265—275

Mies, H. J Einengung von Liquor cerebrospinalis als Vorbereitung zur Papierelektrophorese *Klin. Wschr* 1953. *31* 159—161

Ord, M. G and R. H. S. Thompson Pseudo-cholinesterase activity in the central nervous system. *Biochem. J* 1952. *51* 245—251

Porath, J Purification of bacitracin polypeptide by charcoal chromatography and zone electrophoresis. *Acta chem. scand.* 1954 *8* 1813—1826.

Rees, M. and R. E. Hinsvark, Cholinesterase activity in cerebrospinal fluid. *Nature* (Lond. 1948. *161* 18.

Sober, H. A., F J Gutter, M. M. Wyckoff and E. A. Peterson, Chromatography of proteins II Fractionation of serum protein on anion-exchange cellulose *J Amer chem. Soc* 1956 *78* 756—763.

Svensmark, O Localization of cholinesterases in the protein fractions of cerebrospinal fluid Abstr IV in *Congr Biochem* Wien. 1958. p *171*

Svensmark, O Effect of sialidase on the electrophoretic properties of human serum cholinesterase *Danish med. Bull* 1961 a. *8* 28—29.

Svensmark, O Human serum cholinesterase as sialo-protein. *Acta physiol. scand.* 1961 b *52* 267—275

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Distribution of Blood in the Arousing Hibernator

By

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Abstract

Johansen, K. *Distribution of blood in the arousing hibernator* Acta physiol. scand. 1961 *52*. 379—386. — The distribution of circulating blood has been studied in the arousing hibernator following the method of fractional distribution of radioactive indicators (Sapirstein 1958) The results indicate a conspicuous differential vasoconstriction of the posterior part of the animal during the most active phase of arousal. The blood flow to skeletal muscle in the front of the arousing animals is more than 16 times greater than in the awake non-hibernating animals. The perfusion rat to the myocardium is twice as large in the arousing animal. Likewise the lungs, diaphragm, and brown fat show substantial increase in blood flow Blood flow to gastrointestinal tissues is greatly reduced in the arousing animals, whereas flow to the skin, liver and thyroid shows insignificant differences.

During the last 20 years papers on the physiology of hibernation in mammals have been published unabatedly It has, however not been possible to study experimentally many facets of this profound phenomenon because the instrumentation and techniques available have disturbed the object of study This has been particularly true for the study of dynamical cardiovascular physiology in the hibernating and arousing animal. Great progress was made in this field when Lyman *et al* (1960) pplied the implantation technique for vascular catheters devised by Still *et al.* (1956) and made successful recordings of blood pressure and pulse pressure in ground squirrels during hibernation and arousal from hibernation. The dramatic and physiologically very active process of arousal from hibernation involves profound changes in cardiovascular parameters like heart rate, blood flow blood pressure, peripheral

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resistance, etc. Moreover it has been assumed that *extensive changes in the* distribution of the circulating blood takes place. This is thought particularly to involve massive constriction of the vascular bed in the caudal part of the animal in the earlier part of the arousal process. Evidence for this assumption was claimed early by MARÈS (1892) and DUBOIS (1896) using dye injections in an effort to map the distribution of the circulating blood. A number of investigators (LYMAN and CHATFIELD 1950 JOHNSON 1930 BENEDICT and LEE 1938, JOHANSEN and KROG 1959) have tried to demonstrate a differential vasoconstriction by multiple temperature measurements. These studies have confirmed the idea that the front part of the arousing hibernator warms more rapidly than the hind part. In 1950 LYMAN and CHATFIELD demonstrated that the circulating blood *was distributed in accordance with this temperature* difference. They injected a radio-opaque material directly into the heart and followed its distribution with X rays. In normal anesthetized animals the injection of Thorotrast outlined, in these authors view the whole circulatory system, whereas in the arousing animal only the cranial portion of the circulatory system was visible on the X-rays. All these studies have limitations and are in the author's mind only qualitatively indicative of the distribution of blood. Differences in organ blood flow as well as comparisons of perfusion rates between front and hind portions of the animals are impossible to evaluate with these methods. There seems thus to be no study available which shows quantitatively the distribution of blood in the arousing hibernator The present study utilizes the method designed by SAPIRSTEIN (1958) *for measuring regional* blood flow by fractional distribution of radioactive indicators. Comparison is made between one group of awake animals and one group of arousing animals.

Material and Methods

Arctic ground squirrels, Spermophilus undulatus, were used. They were *housed* individually in wired cages placed at an ambient temperature ranging from *5—10*. Their diet consisted of fresh vegetables, fruit and Fritoes cubes. This species, like most other hibernators, hibernates for shorter periods between which it is awak. Two groups of animals were used. One group consisted of animals that had been awake for at least four days consecutively The other group consisted of animals that had been *in hibernation for minimum of three days.*

The method used for measuring the distribution of blood is based on the *fact that* the uptake of an indicator by an organ is related to the organ blood flow the arterial concentration of the indicator and the organ's extraction ratio for the *indicator*. SAPIRSTEIN, studying rats and using K^{42} and Rb^{86} as indicators, found *that the organ* indicator content became constant 9 sec after intravenous injection and *stayed constant* up to 64 sec after injection. He found a uniform extraction ratio for all organs during the first 64 sec. It is emphasized that the brain is the *only organ showing deviating* results from this and is consequently not included in the *present study. In the present* study only relative alues were sought and onsequently no integrations done of the arterial concentrations after injection. *For details about the method the reader is referred* to SAPIRSTEIN (1958)

The awake ground squirrels were anesthetized lightly with Pentobarbital Sodium by the intraperitoneal route. The femoral vein was exposed and cannulated with polyethylene catheter PE 50. Five μc of $Rb^{86}Cl$ in 0.5 ml saline was then injected and the catheter flushed with heparinized saline. Thirty seconds after injection the animal was killed by injecting 1 ml of a saturated sol tion of KCl which stopped the heart instantaneously Postmortem blood was withdrawn for determination of re maining activity Subsequently the animal was dissected and tissues excised, weighed and counted for activity For counting, a Nuclear Chicago welltype scintillation counter was used, together with Nuclear Chicago scaler Tissues were counted for 5 min at 1 100 volts.

The hibernating animals were moved undisturbed to the operating table. The femoral vein was quickly exposed and cannulated with polyethylene catheter The operational procedure lasted from 2—4 min and initiated an arousal in the animal. By the end of the operation the body temperature of the animal had usually risen to 8—10° The animal was then left in an undisturbed position. The process of arousal continued and when the rectal temperature had reached 15 the isotope was injected and the animal killed as previously described. It was pparent t this time that the process of arousal had entered its most active phase. The oral temperature was more than 15 higher than the rectal temperature. Violent bursts of shivering and muscular ac tivity took place in the front part whereas the hind part was shivering only slightly

Results

Table I summarizes the results. Th activity is expressed as counts per gram tissue per minute or as percent activity recovered of the njected dose. The values are average for 4 or 5 animals in each group

The most conspicuous difference between the arousing and the awake animals seems to be in the skeletal muscle. In the awake anesthetized animals there was a slightly higher activity in the front than in the hind leg muscles. In the arousing animals, however the difference was sixfold and more remark able the activity of the front leg muscles in the arousing animals was more than 16 times greater than in the awake anesthetized animal. The hind leg muscles of the arousing animals showed somewhat higher activity than the hind leg muscles in the awake animals. This difference was probably related to a slight muscle shivering starting also in the hind part of the arousing animal.

The activity of cardiac muscle from the left ventricle in the two groups was more than twice as great in the arousing animals. This difference was significant to the one percent level and indicates a large increase in coronary perfusion during arousal. The lungs as well as the diaphragm likewise showed a substantially higher activity in the arousing animals. The needs for an in creased circulation to these sites is apparent when watching the violent respiratory efforts in an arousing hibernator The skin taken from the footpads showed a very low activity in both groups, suggesting a general peripheral vasoconstriction during the process of arousal. The ctivity of tissues from th gastrointestinal system in the two groups demonstrated a markedly reduced

Table I The table summarizes the results. The activity is expressed as counts per gram tissue per minute or as percent activity recovered of the injected dose. The values are averages for 4 or 5 animals in each group. The data has been treated statistically using the method of Siegel

(S. Siegel, Non-Parametric Statistics, New York, McGraw-Hill, 1956)

Organ or tissue	Arousing animals		Awake animals		
	Counts/g min.	Percent of inj. dose	Counts/g min.	Percent of inj. dose	P value
Skeletal muscle front part	6,532	2.27	393	0.14	P < .01
Skeletal muscle hind part	779	0.27	263	0.09	P < .05
Skin-front legs	630	0.22	504	0.18	N.S.
Skin-hind legs	446	0.16	446	0.16	N.S.
Heart-ventricular myocardium	16,442	5.73	7,850	2.73	P < .01
Diaphragm	8,287	2.89	2,373	0.83	P < .05
Kidney	11,700	4.09	33,116	11.55	P < .05
Stomach	852	0.30	3,763	1.31	P < .01
Small intestine	243	0.08	3,834	1.34	P < .01
Liver	2,211	0.77	2,330	0.81	N.S.
Lung	13,936	4.86	5,835	2.03	P < .05
Brown fat	10,850	3.78	1,812	0.63	P < .01
Thyroid	5,008	1.75	1,872	0.66	N.S.

activity to these areas in the arousing hibernator. Thus the stomach showed 1/4 the activity of the awake animals. The small intestines in the awake animals similarly showed 15 times more activity than in the arousing animals. The same trend, although much smaller, was also apparent comparing the kidney activities in the two groups. The liver and the thyroid showed small and non-significant differences in their activities comparing the two groups. Of great interest, however, the brown fat excised from the scapular region in the two groups showed more than 5 times as much activity in the arousing animals.

Discussion

The method used in this study is based on the fact that the uptake of indicators by an organ is determined by the integrated arterial concentration of the indicator, the volume flow of blood to the organ, and the organ's extraction ratio for the indicator. We have thus presumably three variable parameters and, when measuring only organ activity, a variance in any of the three parameters would influence the results. In our case, using Rb^{86} as an indicator, it turned out that the indicator content of the various organs stayed constant between 9 to 70 sec after injection of the isotope. The brain was the only organ showing deviating results. Indirectly, this means that every organ has the same extracting ratio within these time limits. If this were not the case

the recirculating isotope (between 5—10 %) would change the activity in such a way that organs with high extraction ratios would accumulate isotope while organs with low extraction ratios would give it up. In the present study the samples of animals were as much as possible selected on a uniform weight basis. This fact, together with careful dosage of the indicator justifies the assumption of a reproducible arterial concentration of the indicator. It is emphasized, however that no extensive measurements were made of the integrated arterial isotope content. Thus the data do not give numerical values of organ blood flow. The data is, however representative for relative changes in blood flow to organs and tissues.

The results give ample confirmation to earlier findings of a differential distribution of blood between the front and hind part in the arousing hibernator. Earlier evidence for this condition was derived from temperature measurements and more directly from the radiological studies of LYMAN and CHATFIELD (1950). The early studies of Dubois (1896) and MARES (1892) using intravascular injections of various dyes (Indigo carmine) indicated that circulation primarily took place in the anterior portion of the animal.

The introduction of a radio-opaque material directly into the heart is technically extremely difficult. In order to get reproducible results the contrast material should be injected in the same heart compartment and under the same pressure in each animal. The difficulty involved in this in an animal the size of a hamster can be easily appreciated. Th interesting results presented by Lyman and Chatfield must necessarily be interpreted with these limitations in mind. The X ray pictures reproduced in their article show a clear delineation of the great thoracic veins 4 seconds after contrast injection in the hibernating animal. This is contradictory to their finding of a systemic circulation time of 10 seconds in the awake animals with many times faster circulation.

One of the more conspicuous characteristics of the hibernators is their ability to arouse themselves from the hibernating condition. The speed nd control with which this arousing process takes place have been subjected to repeated investigation by workers in this field. In the small birchmouse (7—16 g) the whole process of arousal takes place in about half an hour and involves a rise in B. T. of 32° (JOHANSEN and KROG 1959). In other animals studied the arousal takes longer time than this, but is always characterized by a rapid warming of the front part anterior to the diaphragm and later rewarming of the posterior parts. Many studies have been designed to elucidate the possible sources of heat and the control of the physiological efforts on the part of th animal that makes possible this profound and dramatic phenomenon.

There are two advantages immediately apparent in regard to the selective distribution of blood during arousal. Firstly it reduces the amount of tissue to be warmed and the surface area for heat dissipation. Secondly it renders possibl a maximum blood flow to organs thought to be involved in the thermogenesis. The outstanding work of DUBOIS (1896) initiated the interest in this

field. His approach was to eviscerate parts of the animals and to study the effects on the awakening process. He also did experiments clamping the aorta or ligating the coeliac trunk and superior and inferior mesenteric arteries. These manipulations did not change the warming pattern of the anterior portion of the body. Tying the vena cava above the liver or tying the portal vein did slow the warming process. Dubois concluded from his experiments that the liver was the predominant source of heat during the warming process. Later studies with evisceration techniques and multiple temperature measurements (LYMAN and CHATFIELD 1950) have questioned the importance of the liver for thermogenesis in the arousing hibernator. The impaired warming after acute portal stasis has later been attributed to pooling of blood in the splanchnic bed rather than interference with the liver as a heat source (BOLLMANN and MANN 1936). The results arrived at in the present study showed no significant change in liver blood flow comparing the awake and the arousing hibernator. This finding supports LYMAN and CHATFIELD's conclusion that the liver has no significant importance as a heat source in the immediate warming process.

The skeletal muscle mass was an early suspect as a source of heat in the arousal process. Thus action potentials have been recorded from muscles in the anterior part at very low temperatures and even before noticeable muscular movements were apparent (LYMAN and CHATFIELD 1950, ANDERSEN, JOHANSEN and KROG 1960). Generally the muscular shivering gets increasingly more violent in the front part until at oral temperatures of more than 30° C the hind leg muscles also come into play. DUBOIS (1896) and later PEMBREY (1901) and JOHNSON (1929) all demonstrated the importance of muscle shivering for the waking process. This has later been substantiated by evidence from work by Lyman and Chatfield. They demonstrated a sizeable reduction in the speed of rewarming after curarization of their experimental animals. The present study demonstrated a remarkable shift in the circulating blood to the skeletal muscle in the front part of the animals resulting in a perfusion rate more than 16 times greater than in the awake animals. It is interesting to note that BENEDICT and LEE (1938) reported that muscle shivering during arousal in the marmot produced more energy than when awake animals were exercised to the limit of their endurance. DUBOIS (1896) stated that the contractions of the diaphragm were of utmost importance in the warming process. JOHNSON (1929) working with the thirteen lined ground squirrel (*Citellus tridecemlineatus*) concluded that the main heat source was the heart and the respiratory muscles. The present data demonstrates a more than threefold increase in perfusion to the diaphragm of the arousing animals. The high activity found in the lungs further substantiates the importance of the increased respiratory efforts in connection with the rapid increase in heat production. Since the lungs represent the only tissue of probable importance for extraction of indicator in the pulmonary circuit, an increase in activity of the

lung parenchyma is suggestive of an increase in cardiac output during the process of arousal.

Special interest is related to the heart as a possible source of heat production during arousal. This muscle is contracting maximally at speeds up to twice the rate ever seen in an awake condition. From the standpoint of rapid transport of heat to adjacent areas the heart and lungs are the only organs in immediate contact with the total cardiac output. On this basis it seems of particular importance that the present study demonstrates a doubling of the activity in the cardiac muscle in the arousing animals. LYMAN and CHATFIELD (1950) apply some interesting logic to the importance of the heart as a heat source during arousal. The conditions prevailing at this time render the heart rather inefficient as a pump leading to particularly high transfer of energy to heat. The particular conditions favoring this are the extremely fast heart rate together with a high peripheral resistance and the high blood viscosity.

The profound changes in blood flow that occur in the arousing hibernator require that blood flow to some sites normally at high perfusion rates must be markedly reduced during arousal. The finding in the present study of a large reduction of the circulation to the gastrointestinal organs seems of special significance in this connection. The 15-times reduction in blood flow to the gut usually receiving 15—20 of the cardiac output represents a major change in the circulatory pattern.

The tissue most often designated as brown fat has from time to time been suggested to play a vital role in the process of hibernation. The finding of a more than 5 times increase in perfusion rate of this tissue in the arousing animals as compared with the awake animals seems very important. The brown fat in the scapular region was shown in this study to have a blood flow more than 150 times higher than ordinary white fat (omental fat). In addition to the rich vascular supply the brown fat is richly innervated. The observation that the thyroid shows rather insignificant increase in blood flow compared with the brown fat may be indicative of a crucial role in the arousing process.

There is yet no evidence available pertaining to the control of the conspicuous shifts in blood flow that occur during arousal from hibernation. The present results strongly suggest that the changes in resistance to flow in the various organs are set up locally. The difference in activity of the thyroid versus the brown fat which are largely supplied by the same main arteries serves to illustrate that intrinsic regulation of vascular resistance at the organ or tissue level is the probable effector mechanism for the great shifts in blood flow. The tremendous differences taking place in skeletal muscle should prove interesting from the point of view of arteriolar shunting mechanisms. How these local changes in peripheral resistance are controlled and monitored by the nervous system to accomplish the unique process of arousal from hibernation is completely unknown.

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INDEX AUCTORUM